Factors affecting the output of hormones

by the adrenal gland:

SECRETORY CAPACITY AND HORMONE STORES OF THE ADRENAL CORTEX UNDER DIFFERENT EXPERIMENTAL CONDITIONS

by

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CHAPTER 1

Introduction

The work embodied in the presented thesis forms part of investigations carried out in this laboratory in order to obtain information about factors which influence the hormone output from the mammalian adrenal gland *in vivo*.

Experiments will be reported which were performed in order to measure the secretory activity of the adrenal cortex under different conditions of stress and under the influence of drugs. The approach used was the direct chemical estimation of the corticosteroids secreted into adrenal vein blood and stored in adrenal glands.

1. Historical preface

Research on the adrenal cortex developed along the classical lines of endocrinology. The interest in the physiological function of this organ emerged from correlations observed between pathological changes in the adrenal gland and certain clinical symptoms (Sampson, 1697; Addison, 1849; 1855). The fact that the gland is essential

for life was suggested by Addison's classical observations. Experimentally the same result was obtained by Brown-Séquard (1856) who described the fatal effect of bilateral adrenalectomy. His experimental technique was, however, so crude that his experiments are quite inconclusive. The same conclusion was, however, reached in 1891 by Abelous and Langlois in their work on the frog. Wheeler's and Vincent's (~~1897~~ 1917) observation that adrenal demedullation did not lead to any serious symptoms directed the interest to the adrenal cortex as a vital organ. The next step was the preparation of extracts from adrenal tissue which prolonged the life of adrenalectomized animals (Hartman et al. 1927; Goldzieher, 1928 and Swingle and Pfiffner, 1929, 1930). The fact that the hormones are really secreted into the blood was only established when Vogt (1943) found that adrenal vein blood possesses high cortical activity.

The chemical nature of the hormones synthesized and secreted by the adrenal cortex was subject to investigations of several groups of chemists associated with Kendall, Reichstein and Wintersteiner. As a result of their work twenty-eight steroids were isolated from adrenal tissue and obtained in crystalline form. Six of them, namely desoxycorticosterone, corticosterone, 11-dehydro-

corticosterone, 11-desoxycortisone, cortisol and cortisone were eventually recognised as having typical adrenocortical activity by the tests then available. The enigma of the disproportionally high biological activity of the remaining amorphous fraction was only solved in 1954 by the isolation of the important sodium retaining steroid aldosterone (Simpson et al. 1954).

Hypophyseal control of the adrenal cortex was established in the work of Smith and Forster (1926). Extracts of the anterior pituitary with adrenocorticotrophic activity were first obtained in 1933, (Evans, 1933; Houssay et al. 1933; Collip et al. 1933). Research in this field reached a summit with the structure analysis of α- and β-corticotrophin carried out independently by Bell and his collaborators (see Bell and Shepherd, 1955) and by Li and his collaborators (see Li et al. 1955).

2. Current experimental work on the adrenal cortex

Three main lines of experimental work on the adrenal cortex are followed up at present.

The first line is concerned with the action, the mode of action and the fate of corticosteroids

in the body. Attempts are made to synthesize new, more powerful steroids.

The second one is concerned with the biosynthesis of corticosteroids. The studies include: Precursors of the actual secretion products, the enzyme systems involved in their synthesis and the factors by which their elaboration can be effected. The problems are mainly approached by in vitro studies on perfused adrenals, adrenal slices and cell free homogenates.

The third line is concerned with the secretion of adrenocortical hormones in the living animal, and the mechanisms by which it is regulated. The only really satisfactory way to obtain information on the secretory performance of the adrenal cortex is the direct estimation of the amounts of corticosteroids secreted into adrenal vein blood during a given period of time. This method is faced with the difficulty of estimating small amounts of corticosteroids in biological material. Before microchemical methods became available, this problem had been mastered only by very few scientists.

To overcome these difficulties, a number of indirect methods have been developed to examine the secretory activity of the adrenal cortex in an intact animal. Some of them are very easy to handle and therefore a large number of research workers

made use of them and very many data have been collected. The methods are mainly based on:

a. Changes in blood and urinary constituents caused by increased amounts of circulating cortical hormones. For example, the fall in lymphocytes (Daugherty and White, 1947) and eosinophils (Thorn et al. 1948) and alterations of the sodium and potassium level in the urine (Harrop et al. 1937; Thorn et al. 1937).

b. Involution of the thymus during cortical hyperactivity (Selye, 1936).

c. Excretion of corticosteroids and their metabolites in the urine (For review see, e.g. Staudinger, 1952).

d. Metabolic changes in the adrenal gland which are associated with increased activity: Fall of adrenal ascorbic acid and cholesterol concentration under conditions of increased blood ACTH-titre (Sayers et al. 1944).

A great deal of our present conceptions on the physiology of the adrenal cortex rests upon deduction from such indirect studies. Although these indirect methods suffer from inherent limitations and are in many ways open to criticism, the results obtained with them taught us which questions

to try and approach by the more efficient direct methods now available.

Direct information on the secretory performance of the adrenal cortex can be obtained in two ways; either by estimation of the amounts of corticosteroids secreted into adrenal vein blood or by estimation of the amount of circulating corticosteroids in peripheral blood.

The latter procedure is limited by the very small amount of these hormones present at any time. The chemical estimation methods utilised, before introduction of chromatography, lacked specificity and gave usually overestimates. For example, the method of Corcoran and Page (1948) which is based on the production of formaldehyde by periodic acid oxidation will also estimate any substance containing a primary or secondary hydroxy ketone or a glycol group. The method of Porter and Silber (1950) in its various modifications is very sensitive, but it estimates 1. only steroids with an α- hydroxy group on C_{17} and 2. also corticoid metabolites reduced in ring A, which are biologically inactive. Thus, for many purposes, this method has to be used in conjunction with chromatographic procedures.

The least ambiguous way to study adrenocortical activity is the direct estimation of the amounts of corticosteroids secreted into adrenal vein blood.

This way was initiated by Vogt (1943), when she measured in different mammalian species the biological activity of adrenal vein blood by means of the Selye-Schenker test (1938). A similar attempt was made by Paschkis et al. (1950) who used the glycogen deposition test (Venning et al. 1946) for the bioassay. The bioassays were, however, elaborate and did not allow to differentiate between individual steroids.

The introduction of micromethods in steroid chemistry, especially their separation by paper chromatography (Zaffaroni, 1950; Bush, 1950) represented an enormous advance in this field of research. It became possible to estimate qualitatively and quantitatively corticosteroids in small volumes of adrenal vein blood. They also offered the possibility to approach the problem of steroid estimation in peripheral blood.

3. Present work

In this thesis, experiments will be reported in which methods of microchemical steroid estimation were utilised for in vivo studies on nature and rate of adrenocortical secretion in different mammalian species. Experiments were carried out under normal conditions and under the influence of ACTH, adrenaline and oestrogens. The experimental

procedures involved an abdominal operation for cannulating the adrenal vein under anaesthesia. Thus the information obtainable is not valid for normal resting conditions.

Furthermore, experiments will be described in which the corticosteroid content of adrenals of different mammalian species was studied. In the rat, the corticosterone stores in the glands were compared with the secretion rate under different conditions of stress and under the influence of drugs.

CHAPTER 2

General Methods

A. Operative Procedure

I. Rats

Adult male Wister rats from four different colonies were used. The sources were University of Edinburgh Small Animal Breeding Station, "The Bush", Miltonbridge (B-strain); Endocrinology Unit, M.R.C. Edinburgh (E-strain); Organon Laboratories, Ltd. Newhouse, Lanarkshire (O-strain); A. Tuck and Son, The Mousery, Rayleigh, Essex (T-strain). Bi-strain stands for Bush rats kept and fed in the Biochemistry Department of the Edinburgh University, Es-strain for rats received in summer 1955 and Ew-strain in winter 1955/56 from the Endocrinology Unit. They were kept on a diet of rat cake nuts (24.8% protein, 4.7% fat and 49.3% starch; vitamins added q.s.; see Lane-Petter and Dyer, 1952).

1. Collection of adrenal blood

The collection of adrenal vein blood was done in urethane- or in pentobarbitone- anaesthesia (1.5g/kg b. wt. s.c. as 25% solution in 0.9% saline and 45 mg/kg b. wt. i.p. as 1% solution respectively). In addition, ether was sometimes required.

Dissections were done on a heated operating board. The trachea and the right femoral vein were cannulated; the abdomen was opened by a midline incision, the left renal vein dissected, and the renal pedicle tied. A loose ligature was put round the entry of the renal vein into the vena cava and all affluents to the renal vein, not coming from the adrenal gland, were tied. Heparin (1000 i.u./kg) was injected via the cannula in the femoral vein and a ligature placed around the renal vein lateral to the entry of the adrenal vein. The ligature at the mouth of the renal vein was tied, and a long polythene cannula, previously passed through the flank of the rat, inserted into the renal vein. The abdomen was then closed. Adrenal vein blood was collected over a period of 15 to 30 minutes in a siliconed glass tube immersed in ice water. Rectal temperature was taken throughout the blood collection. Blood pressure was recorded from a cannula in the right femoral artery connected to a Condon mercury manometer (Condon, 1953). The cannula in the femoral vein was connected to a small burette, from which saline or blood could be infused, in order to maintain the blood pressure constant during the blood collection. The blood used for infusion was taken from a cannula in the carotid artery of a heparinised donor rat in ether anaesthesia. It was gently shaken in a water

bath at 37°C. Small volumes were transferred to the burette immediately before administration. Drugs were infused with a Palmer's slow injection apparatus.

2. Experiments on adrenal glands

For experiments on "unstressed rats" the animals were kept single and absolutely undisturbed in a temperature-regulated room (25°C). They were killed by rapid decapitation in the same room. The glands were dissected and either worked up immediately or kept in a deep freeze at -16°C for not longer than 24 hours. "Stressed rats" were bled under ether anaesthesia from the cannulated carotid artery after heparin (1000 i.u./kg) had been injected. The whole procedure lasted approximately 15 minutes.

3. Demedullation of adrenal glands

The adrenal glands were exposed under ether via the dorsal route and enucleated. For the first week after the operation the rats received 0.6% NaCl solution instead of drinking water. At the end of each experiment the regenerates were investigated histologically.

II. Larger Animals

The operative procedures in monkey, cat and dog were essentially the same. These experiments were performed to study the influence of adrenaline

on the function of the adrenal cortex. To exclude interference from endogenous medullary amines both adrenal glands were deprived of their sympathetic nerve supply in a preceding operation under aseptic conditions (atropine ether, abdomen opened by a midline incision, both splanchnic nerves severed and the first three lumbar sympathetic ganglia extirpated on each side, abdomen closed by a muscle and a skin suture). The number of days left for recovery varied. Collection of suprarenal vein blood was done as described by Vogt (1943). Dissection was started in ether, the trachea and the left and right femoral veins were cannulated and chloralose (70-80 mg/kg, 1% solution in saline) slowly injected intravenously. The right femoral artery was cannulated and connected to a mercury manometer for blood pressure records. The abdomen was opened and, depending on the anatomy of the individual animal, either the lumbo-adrenal vein or the renal vein dissected. In the latter case, the arterial blood supply to the kidney was interrupted before the renal vein was tied at its origin. A loose ligature was placed round the entry of the renal or the lumbo-adrenal vein into the vena cava and all tributaries to these veins, not coming from the adrenal gland, tied. 1000 i.u. heparin were injected and a

cannula inserted into the vein carrying the adrenal outflow. This cannula was connected to one arm of a T-piece by a short portex tubing passed through the flank of the animal. The stem of the T-piece was connected by another portex tubing to the cannula in the left femoral vein. The loose ligature around the caval junction of the vein was tied and suprarenal blood could now either be drained from the second arm of the T-piece into a siliconed, cooled glass tube by clamping the tubing which led to the femoral vein, or could be directed back into the animal by occluding the second arm of the T-piece. For intra-arterial injections a double cannula with a side arm, closed by a piece of rubber tubing, was introduced in the central and peripheral end of a cut in the left carotid artery, so that the blood flow to the brain was not interrupted.

For intraventricular injections in cats a parasagittal incision was made over the parietal bone and a trephine hole drilled through it at a point where the insertion of the temporal muscle curves towards the midline. Its centre was 6-7 mm from the midline and 14-15 mm in front of the external auditory meatus (Feldberg and Sherwood, 1953). In monkeys, the centre of the trephine hole lay 10 mm behind the coronal suture and 6 mm lateral to the midline. In the dog, the insertion of the temporal

muscle was dissected off and a trephine hole made with its centre 7 mm ~~dorsal~~ posterior to the coronaty suture and 7 mm lateral to the midline.

The red cells in the suprarenal blood samples were centrifuged off immediately after collection and the plasma subjected to the chemical estimation procedure outlined below.

B. Procedure for Qualitative and Quantitative Chemical Estimation

The chemical estimation method for corticosteroids in adrenal vein blood and adrenal glands consisted in extraction of the steroids, purification of the extracts and separation of the individual compounds by chromatography on paper. For qualitative purposes a soda-fluorescence test with "Blue Tetrazolium" in the sodium hydroxide was carried out on the paper. For quantitative estimations the paper regions containing the corticosteroid spots were eluted and the intensity of the reaction between the eluates and "Blue Tetrazolium" measured.

I. Reagents

The following reagents were used in the course of the estimation procedure:

Blue Tetrazolium (BT): B.D.H. 3,3'-dianisole bis-4,4'(3,5-diphenyl) tetrazolium chloride.

Ethanol: Aldehyde free, prepared by the method devised in U.S. Pharmacopoeia (15th ed., p.954).

The first distillate was distilled a second time. After that treatment the ethanol was 98-99% pure. Ethanol referred to as "95%" was prepared by taking 5 ml of aqua dest. and making up to a 100 ml volume with the 98-99% ethanol.

Ethylacetate: Ethylacetate "analar", B.D.H. was washed once with 1/8 vol. of 1% Na_2Co_3 and twice with 1/8 vol. water, dried over $CaCl_2$ and distilled twice.

Ethylene dichloride: George and Becker, London. Twice distilled.

Ether: Ethyl ether, "analar", B.D.H. Once distilled shortly before use.

Methanol: Distilled from NaOH (5g/l.) and redistilled once without alkali. Refluxing with alkaline tetrazolium did not improve on the final results.

Petrolether: "analar", B.D.H. b.p. 40-60°C.

Tetraethylammoniumhydroxide (TEA): B.D.H. 25% w/w in water.

Tetramethylammoniumhydroxide (TMA): B.D.H. 25% w/w in water. TEA and TMA were titrated before use.

Tetrazolium salt: B.D.H. 2,3,5-triphenyltetrazoliumchloride.

Water: glass distilled water was used.

All other reagents used and not mentioned in this section, were "analar" and not further purified.

Glassware: All test tubes and stoppers used in the elution process and for the reaction with B.T. were boiled twice in distilled water after they had been washed with chromic acid and water.

Chromatography papers: Whatman No.2 papers were used. Washing the papers with organic solvents was tried. The paper blanks rather increased than decreased after such treatment and therefore the procedure was abandoned.

II. Extraction of Corticosteroids and Purification of Extracts

1. Adrenal vein plasma

The method used for the extraction of corticosteroids from adrenal vein plasma was the method described by Bush (1952) with some simplifications, devised by Vogt (1955). The plasma was diluted with an equal amount of water and extracted four times by vigorous shaking with 1 volume of an ethyl acetate-ether mixture (2:1). The supernatant organic phase was pipetted off into a round bottom flask and each portion evaporated to dryness in vacuo at 50°C in the same flask. The dry residue of the combined supernatants was transferred into a separating funnel by washing the flask with 2.5 volumes (but not less than 10 ml) of petrolether, followed three times by 0.5 volume (but not less than 2 ml) 80% ethanol. Each portion of ethanol

was shaken vigorously with the petrolether, drained off and evaporated separately in the same tube. The dry residue was finally taken up in 0.4 ml ethylene dichloride and applied on paper. Two further portions of 0.2 ml ethylene dichloride were used to complete the transfer of the steroids to the paper.

2. Adrenal glands

Because of the high concentration of salts and phospholipids in adrenal gland tissue a more elaborate purification procedure proved necessary. The glands were dissected free from adhesive fat and connective tissue and weighed on a micro-balance. Depending on the expected corticosteroid concentration 50 to 400 mg of tissue were used. The steroids were extracted by homogenizing with five 2 ml portions of ethylacetate in an all-glass homogenizer and the tissue residues separated from the extract by centrifugation. The precipitate was washed twice with 5 ml ethylacetate. (Microscopic inspection of the precipitate did not show any intact cells. Only a few sudanophilic droplets could be observed). The three combined supernatants were washed in a separating funnel by gentle shaking with consecutive portions of 2.5 ml 0.2N Na_2CO_3, 2.5 ml water and 2.5 ml slightly acidified (pH5) water. The bottom layers were drained off each time. The ethylacetate ex-

TABLE 1

Chromatography systems for separation of individual corticosteroids

I. Adsorption methods

Method	Reference
Elution from alumina columns	Reichstein and Shoppee (1949)
Elution from silica gel columns	Pincus and Romanoff (1950)
Elution from silica gel columns (microcolumns)	Sweat et al. (1953)
Elution from florisil columns	Nelson and Samuels (1952)
Elution from magnesium silicate columns	Nelson and Samuels (1952)
Alumina paper (microcolumn)	Bush (1950), Shull et al. (1952)

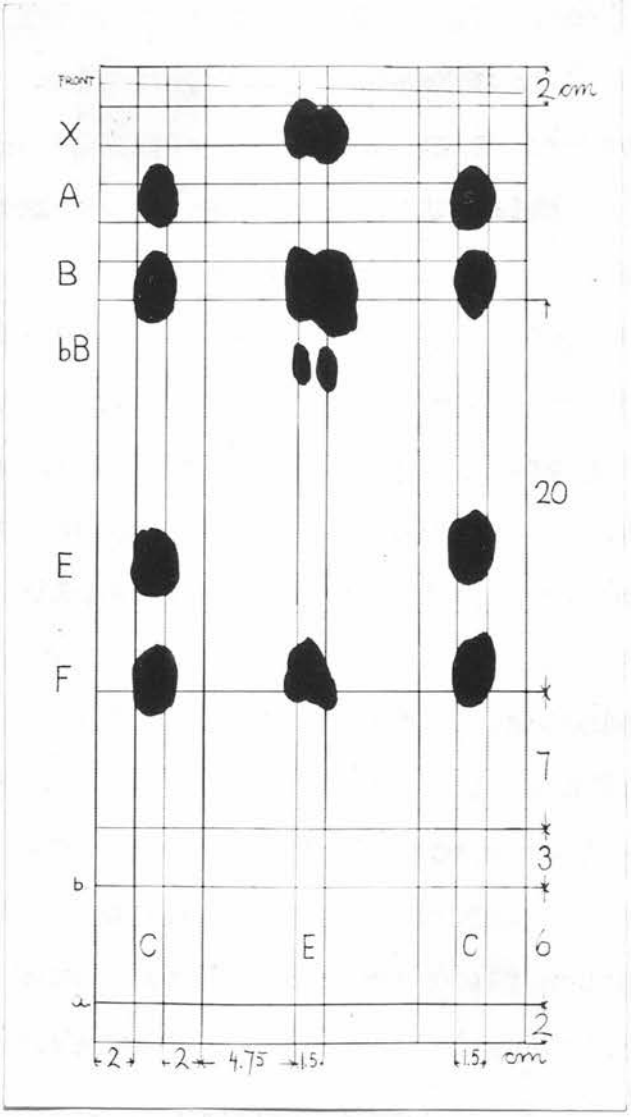
II./

TABLE I Contd:

II. Partition methods

Supporting material	Solvent system		Reference
	mobile phase	stationary phase	
Kieselguhr	Toluene	aqueous ethanol	Morris and Williams (1953a)
	Ethylene glycol	25% light petroleum in toluene changed continuously to 100% ethylene dichloride	Morris and Williams (1953b)
Filter paper	Benzene Toluene	formamide propylene glycol	Zaffaroni et al. (1950)
Filter paper	Benzene	aqueous methanol	Bush (1952)
Filter paper	Water	butanol	Hofmann and Staudinger (1951)
Silica gel	Ethanol or methanol	methylene chloride containing ethanol or methanol	Katzenellenbogen et al. (1952)

Figure I



Paper chromatogram of an extract of rat adrenal vein blood to which compound F was added.

tract was dried over Na_2SO_4, decanted and transferred in small portions into a 30 ml Quickfit and Quartz boiling tube and slowly evaporated at reduced pressure (approx. 160 mm mercury) in a water bath at 50°C. The combined bottom layers from the washing procedure were back extracted with 2.5 ml ethylacetate. This ethylacetate was also used to wash the Na_2SO_4 and then transferred into the Q and Q tube. Na_2SO_4 was washed for a second time with 2.5 ml of ethylacetate and the ethylacetate added into the Q and Q tube. The dried residues of the ethylacetate extracts were taken up in 10 ml acetone and three drops of a saturated ethanolic solution of $MgCl_2$ added. The tube was then kept at -16°C for 15 minutes. The precipitate formed, containing phospholipids, was separated off in the centrifuge and washed once with 5 ml acetone. The combined acetone supernatants were evaporated to dryness in a round bottom flask and the residue freed from fats by distribution between petrolether (b.p. 40-60°C) and 80% ethanol. From this point on, the procedure was the same as for plasma extracts.

III. Chromatography

A great number of chromatography systems for separation of steroids have been described during the last ten years (for review see Bush, 1954). Table 1 gives a short summary of methods especially

efficient in the separation of adrenal steroids. In this work, the method of Bush (1952), which employs orthodox chromatography with volatile substances for the mobile as well as for the stationary phase, was used. The method is reasonably quick and simple and makes it possible to obtain in a given time qualitative and quantitative results on a larger number of samples, as it is demanded in biological work. It achieves good separation of the known corticosteroids of physiological importance present in adrenal vein blood and glands, with the exception of aldosterone.

Sheets of filter paper (Whatman No.2) were mapped as shown in Fig. I. The extract was slowly applied on the central area E (6x1.5 cm), the walls of the tube twice washed with 0.2 ml ethylene-dichloride and the washings also put on paper. In the two side areas C pure corticoids (usually 10 µg of each compound in question as a 0.1% ethanolic solution) were evenly distributed as markers. In order to compress the steroids to narrow bands an ascending chromatogram was run at room temperature with a mixture of ethylacetate-methanol (2:1 v/v) until the solvent front reached line b. In this mixture, all corticosteroids travel with the solvent front. Ultra violet scanning of the papers at this stage shows in the regions of the markers dark blue spots on line b,

with only faint, short tails. There was usually no distinct absorption visible in the region of the extract, but a more or less intense fluorescence, caused by impurities. The final chromatographic procedure was carried out exactly according to Bush's description. The solvent mixture consisted of 550 ml methanol, 450 ml water and 1000 ml benzene (Bush and Sandberg, 1953). The papers were equilibrated in the solvent vapours for at least 8 hours, usually overnight at 34°C in a temperature regulated box. Shorter equilibration times yielded spots with long tails. A descending chromatogram with methanol-water-saturated benzene as mobile phase was run for 3-6 hours at the same temperature. Of the corticosteroids of interest in this work, 17-hydroxycorticosterone has the smallest R_F-value in this system, followed by cortisone, corticosterone and 11-dehydrocorticosterone (see Fig. I). Relatively non-polar compounds as progesterone, desoxycorticosterone and some 17-ketosteroids lie near the solvent front. The exact position of aldosterone in this system has not been estimated to my knowledge. In the system toluene/methanol/water (4:3:1 by vol.) it lies between cortisol and cortisone (Singer and Stack-Dunne, 1955); in the system toluene/ethylacetate/methanol/water (9:1:5:5 by vol.) it has the same R_F value as cortisol (Simpson et al. 1954).

IV. Detection and Identification of the Corticosteroids on the Chromatogram

1. Scanning in front of a lamp emitting ultra violet light of about 240 mμ.

Light of this wavelength is absorbed by corticosteroids containing an α,β-unsaturated keto group in ring A. The steroids appear as dark spots on the paper when present in concentrations of more than 0.5 μg per cm^2, and can thus easily be detected. Their outlines can be marked with pencil. The comparison of the R_F values of the compounds present in the extracts with those of the markers provide some indication of their chemical identity. Since impurities in biological extracts sometimes absorb ultra violet light of a similar wavelength this test cannot by itself be used for identification, unless the steroidal nature of the absorbing substance has been established by other means.

2. Reaction with "Blue tetrazolium" on paper

Corticosteroids containing an α-ketol side chain reduce under alkaline conditions blue tetrazolium (BT) to diformazane. When the dried chromatograms are sprayed with BT solution (12 mg dissolved in 5 ml ethanol and added to 25 ml 5% aqueous NaOH) the corticosteroids become visible as violet spots. About 2 μg/cm^2 of a corticosteroid have to

be present. This reaction is not suitable for quantitative purposes. At a time when no scanner was at our disposal to localise the spots in the chromatograms, the sides of the paper where the markers travelled were cut off along the lines c in Fig. I and treated in this way. The control spots were then used as guidance to locate the corticoid containing regions in the part where the extract had travelled so that they could be cut out and eluted for the quantitative assay. To proceed in this way was permissible, because experiments have shown that the R_F values of pure corticoids added to an extract of peripheral plasma are the same as those of pure corticoids in ethanolic solution.

3. Soda-fluorescence test

For the soda-fluorescence test the dried chromatograms were sprayed with a 14% aqueous solution of NaOH, which contained either 5 mg triphenyltetrazoliumchloride or 3 mg "blue tetrazolium" (BT) per 100 ml. The papers were heated in front of a battery of infra red lamps for 15 minutes. When the paper is quite dry the corticosteroid spots show a bright primrose fluorescence in ultra violet light specific for α,β-unsaturated 3-ketosteroids. Steroids with various substituent groups but containing a 3-hydroxyl group do not give this reaction. Equilinine and oestradiol show a blue fluorescence (Bush, 1952).

The addition of a trace of triphenyltetrazolium-chloride suppresses background fluorescence of the paper. When BT is added to this concentrated NaOH solution the steroids with an α-ketol side chain appear almost immediately as blue spots. By this latter technique it is possible to get information on the structure of ring A and the side chain of the corticosteroid in question in one and the same working procedure. If a constituent of an extract gives these two reactions, this is good evidence that it is an α,β-unsaturated ketone with at least 2 rings and that it is a reducing substance. If this compound has the same polarity as a given pure corticosteroid, its identity can hardly be doubted. In some cases an identification by these means has been confirmed by a number of other methods. This was done, for example, by Bush (1953b) for the cortisol found in dog adrenal vein blood.

The sensitivity of the soda-fluorescence test is high. Quantities of 0.5 µg corticoid per cm^2 are clearly visible. Semiquantitative estimates can be obtained by this method by comparing size and intensity of a fluorescing spot from extracts with those of control spots of known quantity. When however, an extract of adrenal vein blood or glands was divided into two equal portions and its corticoid con-

TABLE 2

Reactions for detection and quantitative estimation of corticosteroids

Method	Substance	Approx. limit of sensitivity (μg)	Reference	
			Detection	Quantitative estimation
Alkaline silver nitrate	α-ketols	5	Zaffaroni et al.(1950)	
Aqueous iodine	cortisone and others	5	Zaffaroni et al.(1950)	
Iodine in petroleum	α,β-unsaturated ketones and others	1-2	Bush (1950)	
Ultra violet light	a,β-unsaturated ketones	5	Haines and Drake (1951) Bush (1952)	Samuels (1947); West et al. (1951); Haines (1952); Edgar (1953a,1953b) Zaffaroni & Burton (1953) and others.
Diphenyltetrazolium chloride	α-ketols	3	Zaffaroni et al.(1951)	Hofmann and Staudinger (1951)
"Blue tetrazolium"	α-ketols (and others)	2	Chen and Tewell (1951)	Mader & Buck (1952) Morris & Williams (1955); Vogt (1955) Weichselbaum and Margraf (1955)
Osmid tetraoxide	α,β-unsaturated ketones	2	Manaro and Zygmuntowicz (1951)	
NaOH-fluorescence	$\triangle^4$-3 ketones	0.25	Bush (1952, 1954)	
Arsenomolybdate	Adrenal steroids	5	Schwarz (1953)	
H_2SO_4-fluorescence	Adrenal steroids	0.1	Sweat and Farrell (1953)	

tent estimated in one portion by the soda fluorescence test, in the other by the quantitative BT-microreaction described below, the differences were sometimes up to 50%. It was particularly difficult to make a correct guess when the spots did not differ so much in size as in intensity. A more laborious technique for applying the steroids is probably required in order to overcome this difficulty. For permanent records the dried NaOH sprayed chromatograms can be photographed in ultra violet light.

A number of other reactions for steroid detection are available (see Bush, 1954), but only the three described above were used in this work.

V. Quantitative Estimation after Chromatographic Separation

When adrenal steroids are present in a reasonable pure state, as it is achieved by purification of the extracts and by chromatography, a number of more or less unspecific reactions given by the steroid in question can be used for quantitative purposes. Quantitative chemical methods devised for steroid estimations are summarized in Table 2.

The question was to choose a method which was highly sensitive and as little as possible interfered with by the paper itself.

There are 3 ways in which the estimations can be

done: (1) reaction and estimation on paper;

(2) reaction on paper, elution of the reaction product from the paper and estimation in the test tube;

(3) elution of the steroid from paper, reaction and estimation in the test tube.

1. Reaction and estimation on paper

As discussed above, quantitative assays by the comparison of the appearance of spots of corticosteroid reaction products on the paper is not satisfactory.

An attempt was made to utilize a more objective technique for estimates on paper.

Different amounts of corisol in ethanolic solution were applied on filter paper (Whatman No.2) and dipped at first into a BT-solution (12 mg dissolved in 5 ml ethanol, added to 25 ml 5% NaOH in H_2O) and then into a 5% solution of acetic acid in 50% ethanol. The papers were dried at room temperature in a dark place and made transparent by treating with liquid paraffin containing brom-naphthalene. The light absorption of the spots was measured in a photometer devised by Grassmann and Hannig (1952) for quantitative protein estimations after fractionation by paper electrophoresis. The paper was moved mm by mm along a photocell and the intensity of the light passing through each section measured. The readings (μA) were plotted against the length of the

strip (mm) and the areas of the curves obtained in this way estimated by the aid of a planimeter. A straight line relationship between the logarithm of the amount of cortisol and the areas was observed. Spots of 5, 8, 10, 12, 15 and 20 µg were investigated. There was however, a rather large variation from experiment to experiment. The results were somewhat improved by using a reaction between the steroid and alkaline $AgNO_3$. One of the sources of error was the irregular transparence of the paper background and this was the main reason for discontinuation of these experiments.

2. Reaction on paper, elution of the reaction product and estimation in the test tube

Hofmann and Staudinger (1951b) have devised a technique involving spraying of the paper with triphenyltetrazoliumchloride and eluting the formed formazanes with tetrahydrofuran for colorimetric estimation. Schwarz (1953) used a similar method with arsenomolybdic acid. We were not successful in eluting formazanes quantitatively from the paper.

3. Elution of the steroid from paper, reaction and estimation in the test tube

The following technique was finally adopted for the elution and steroid estimation in the eluate. Its main points have been described by Vogt

(1955).

a. Elution

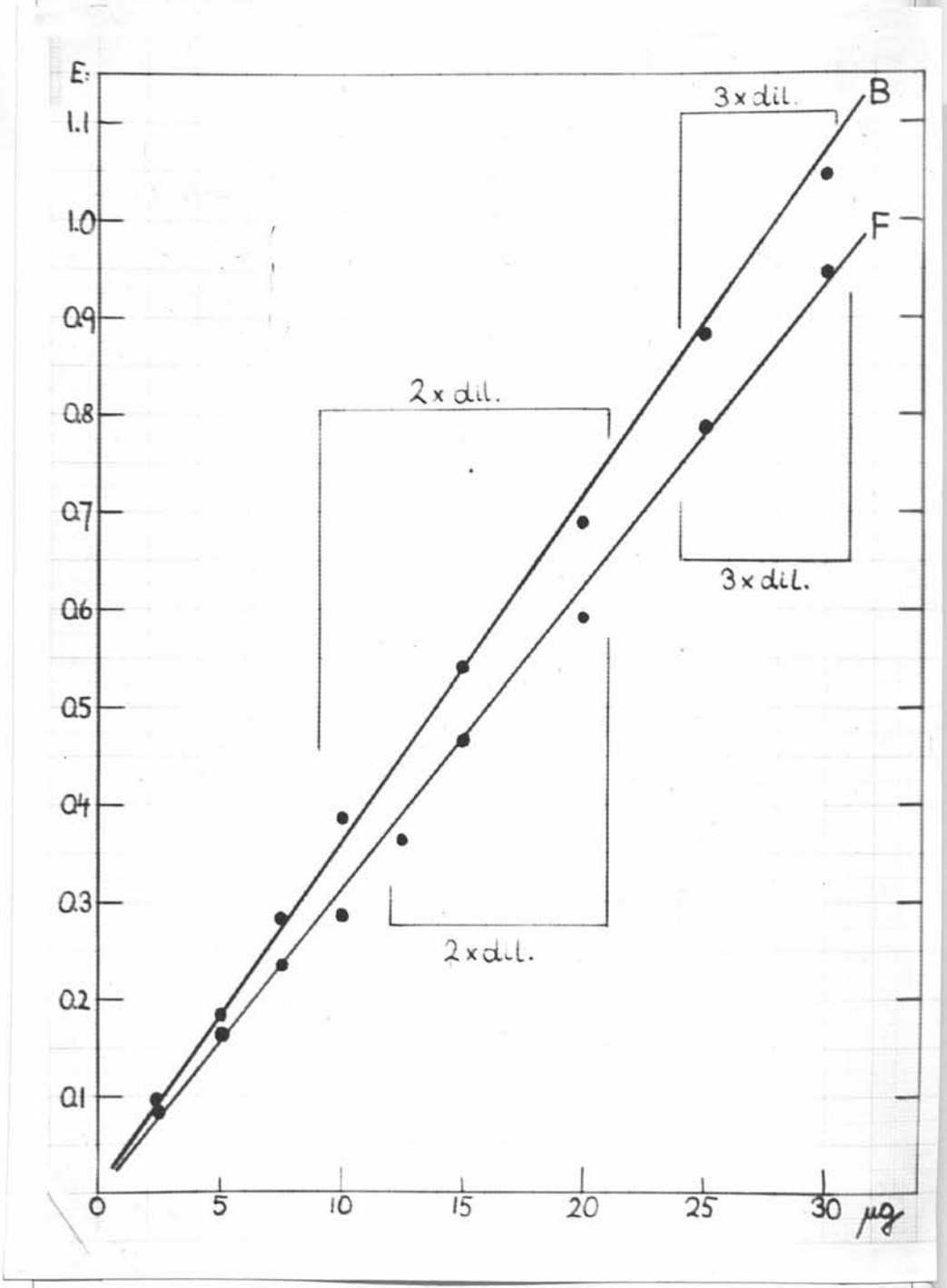
The regions of the paper containing the individual corticoids were cut out. Depending on the size of the spots, rectangles of surface areas amounting to 25, 30 or 36 cm^2 were used. From each paper, areas of the same size were cut out between line c and the region where the extract had travelled to serve as blanks. The rectangles were cut up into small pieces of about 10 mm^2 and eluted with absolute ethanol by shaking vigorously three times for 5 minutes in a microflask shaker. The three volumes of ethanol were 2.7, 1.8 and 1.8 ml when the surface of the rectangles was 36 cm^2; 2.4, 1.5 and 1.5 ml for 30 cm^2, and 2.0, 1.5 and 1.5 ml for 25 cm^2. Each portion was filtered through a small plug of ethanol washed cotton wool, pushed into the stem of a micro-funnel, and evaporated to near dryness at 50°C and approximately 160 mm Hg pressure. The last portion was taken to complete dryness. When evaporation was carried out at lower pressures, low and irregular recoveries were obtained. Another source of error at this stage was the use of rubber to connect the tube to the vacuum pump. Ethanolic extracts of the rubber, which may be sucked back into the sample, spoil the colour reaction with BT. Thick walled polyvinyl tubes were substituted for

the rubber as far as the trap separating the pump from the tube which contained the sample.

b. Estimation

For quantitative estimations the reduction of BT (3,3'-dianisole-bis-4,4'-(3,5-diphenyl) tetrazoliumchloride) to diformazan was chosen. It is given by steroids which contain the primary α-ketol group. The reaction was originally described by Chen and Tewell (1951). They found a straight line relationship between the amount of desoxycorticosterone (5-50 µg/ml) and the colour intensity. Mader and Buck (1952) used this reaction for quantitative assays of larger amounts of cortisone. These authors also observed that compounds containing a 17-OH group develop less colour with BT than compounds lacking this group. The method was adapted by Morris and Williams (1955) for microestimations of corticosteroids in eluates from Kieselguhr columns. Vogt (1955) modified it for eluates from paper chromatograms. As the reaction between steroids and BT is a rather complex one, small changes in pH, water content of the alcohol or temperature lead to variations in the results. The colour also fades on exposure to light. Therefore one has to observe the experimental conditions once chosen, with great accuracy in order to get reproducible results. As the reaction is also given by a number

Figure II



Standard curves for the quantitative estimation of corticosterone and cortisol. Light absorption of the formazans produced with blue tetrazolium. Abscissa: µg steroid/sample; ordinate: extinction. Spekker, Ilford filter 607, micro cells.

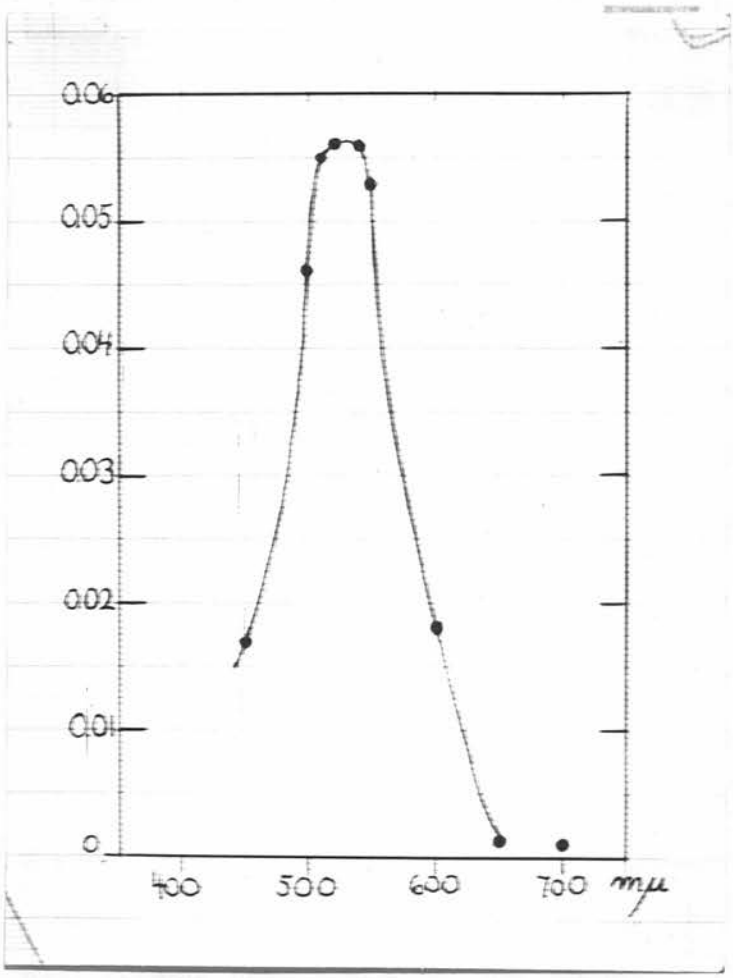
of other reducing substances, it is necessary to take extreme care of the cleanliness of glassware and to the purity of solvents.

The final procedure used in this work was the following: The corticosteroids or the dry eluates from the paper were taken up in 0.45 ml 95% ethanol; 0.04 ml of a 5% solution of tetraethylammoniumhydroxide or of tetramethylammoniumhydroxide in 80% ethanol was added, followed by 0.05 ml of a freshly prepared 0.5% solution of BT in 95% ethanol. The reagents were mixed well, the tubes stoppered and incubated for one hour at 23.5°C in a water bath in the dark. After that 0.04 ml of a 10% solution of acetic acid in 50% ethanol were added. The amount of diformazane formed during the incubation period was estimated by its light absorption. Readings were taken at first in a Spekker absorptiometer and later in a Unicam Spectrophotometer. The threshold of the reaction lies at about 0.5µg/sample.

a. Procedure for the Spekker

After addition of the acid the samples were transferred into micro cells of 0.5 ml capacity and their absorption read against a reagent blank with an orange filter, Ilford 607. In this way, standard cruves for pure corticosterone and cortisol were established (see Fig. II). A straight line relationship between the corticoid concentration in the

Figure III



Absorption curve of the reaction product between cortisol and blue tetrazolium. 5 μg F, final volume: 0.58 ml; micro-cells. Abscissa: wave length; ordinate: extinction. Unicam Spectrophotometer.

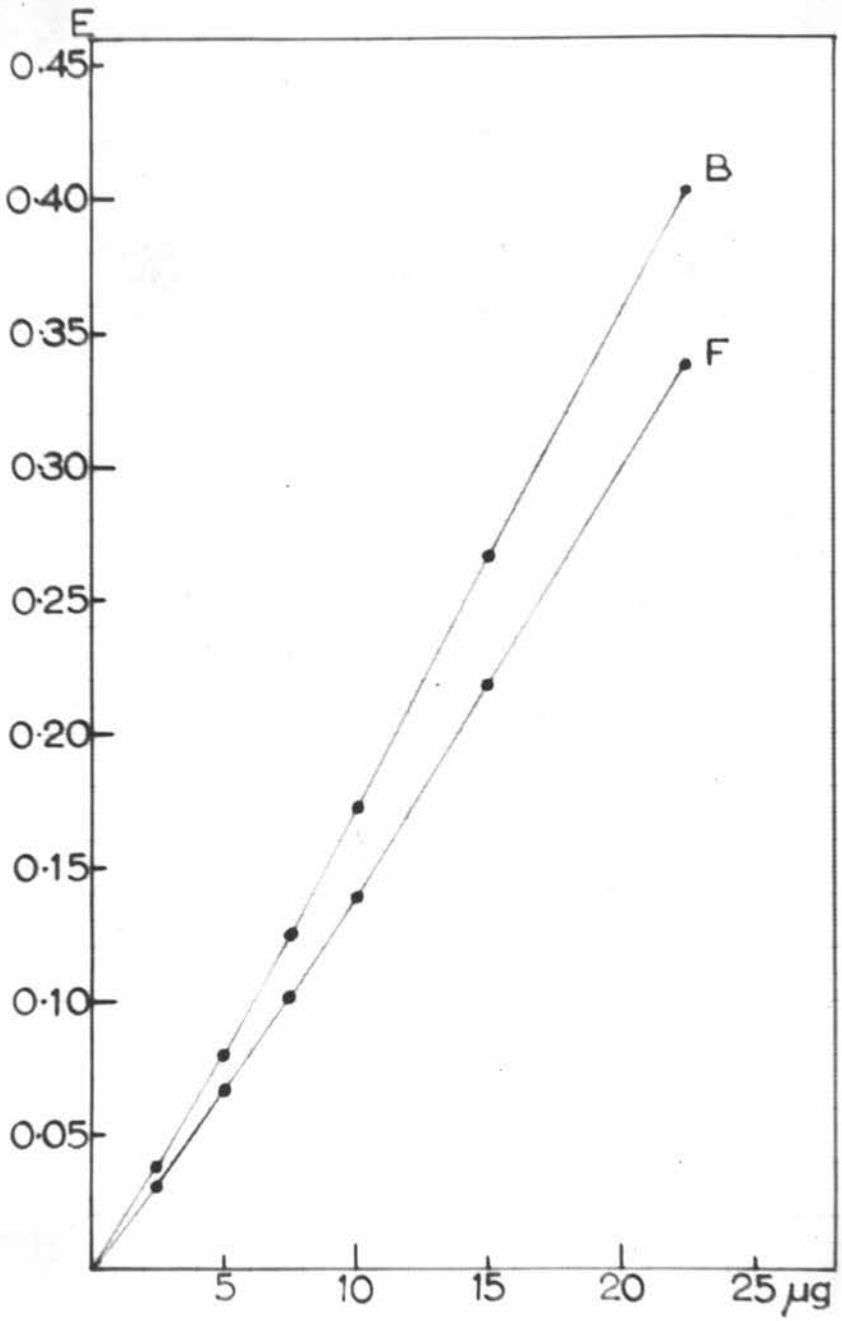
sample and its light absorption was only observed when the extinction did not exceed 0.3. When it was greater than that, 1:1 or 1:2 dilutions of the samples and the blanks had to be used. Under those conditions the readings followed Beer's Law in a region from 2.5 µg to 30 µg of the two steroids. The data in Fig. II represent means of 4-8 readings. Some of them show a deviation from the straight line, which is usually not larger than 5%. Chekcs on the standard curve were made at regular intervals. The reproducibility was satisfactory.

The samples eluted from the paper were read against their own paper blank. Paper blanks were regarded as "normal" when their readings lay between 0.08 and 0.11. This procedure was used in all the experiments done on monkey, dog, cat and rabbit in the rat blood experiments 1 to 123 and in the rat gland experiments I to XVII.

β. Procedure for Unicam Spectrophotometer

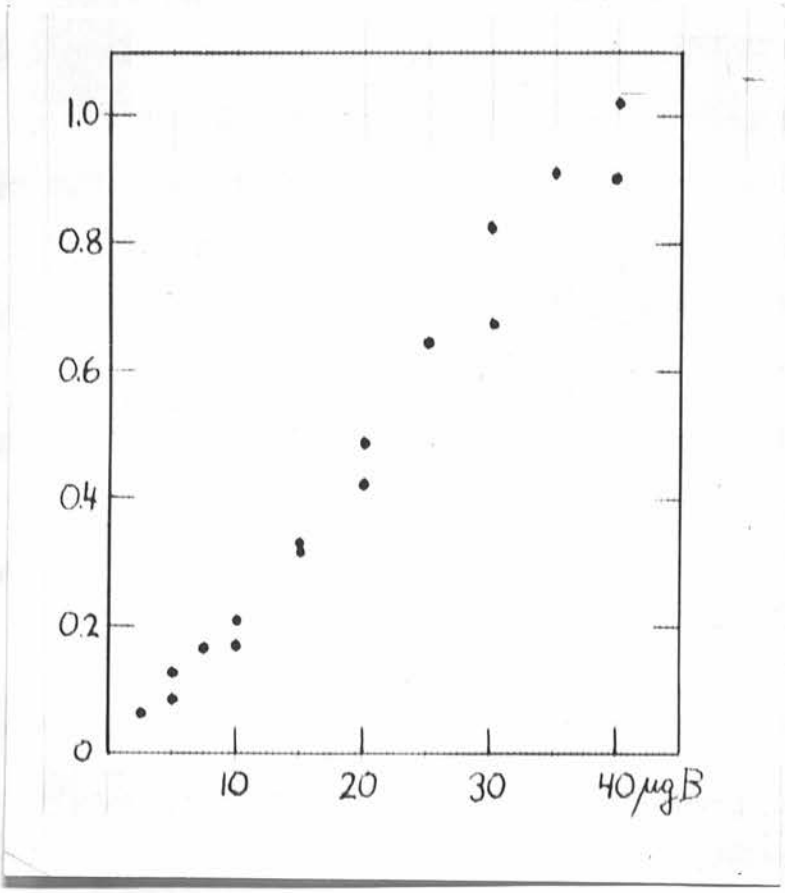
A solution containing 20 µg corticosterone was incubated with BT in the described way. After the addition of the acid 4.42 ml "95" ethanol were added, the samples transferred into a 1x1x4.5 cm glass cuvette and the light absorption measured against ethanol in the photometer at different wavelengths. The peak of the light absorption was estimated to be at 520 mµ (see Fig. III) and all further samples were

Figure IV



Standard curves for the quantitative estimation of corticosterone and cortisol. Absorption at 520 mµ of formazan formed with blue tetrazolium. Abscissa: µg steroid/sample; ordinate: extinction. Unicam Spectrophotometer, macro-cells (1 cm light path).

Figure V



Relationship between corticosterone concentration and light absorption. Method of Weichselbaum and Margraf.

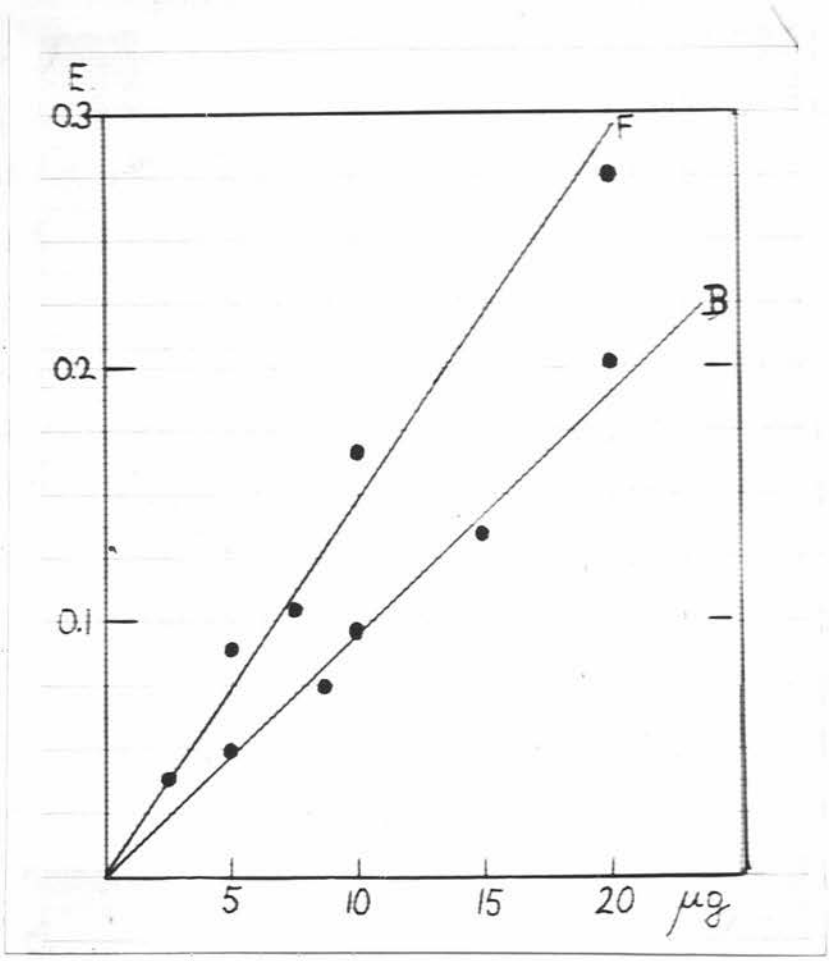
read at this wavelength. To simplify the procedure, only 4 ml of "95" ethanol were later on added to the acidified samples. Standard curves for cortisol and corticosterone were obtained with this volume (see Fig.IV). The curves do not exactly follow Beer's Law. They were however, accurately reproducible. Deviations from the standard curve were smaller than in the Spekker. Dilutions at higher concentrations were unnecessary. The experiments on rat adrenal vein blood from 124 onwards and on rat adrenal glands from XVIII onwards were done by this procedure.

Reagent blanks gave readings between 0.018 and 0.022; paper blanks were regarded as normal when they did not exceed 0.05 for 36 cm^2 rectangles and 0.04 for 25 cm^2 rectangles.

c. Other possibilities for quantitative corticosteroid estimation in paper eluates which were examined.

Weichselbaum and Margraf (1955) used another modification of Mader and Buck's BT test. The corticosteroids were dissolved in 0.3 ml absolute ethanol, 0.1 ml of a freshly prepared solution of BT was added and the incubation continued for another 20 minutes. With this procedure we obtained the figures shown in Fig.V. It does not seem that this

Figure VI

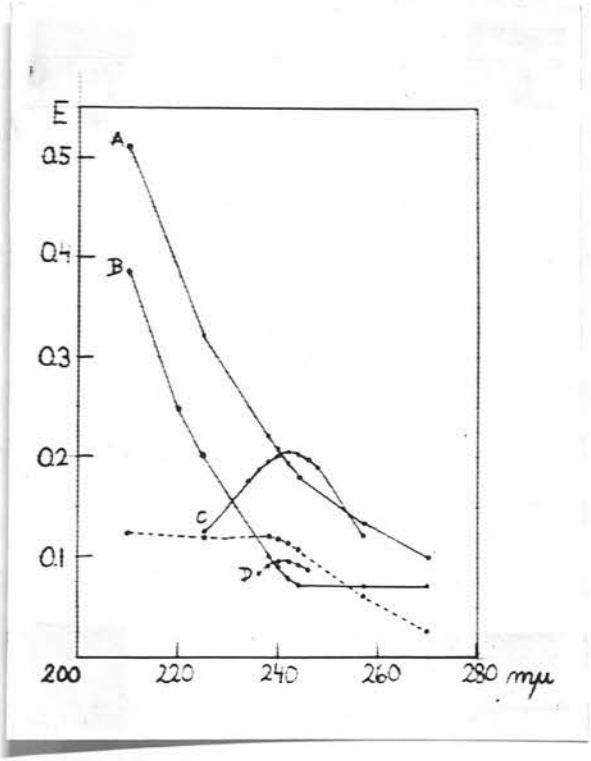


Absorption of ultra violet light (242 mµ) by cortisol (F) and corticosterone (B) in ethanolic solution. Abscissa: µg steroid per sample. Ordinate: extinction.

method offers any special advantages over the method used by us.

A standard curve has also been established by measuring the absorption of ultra violet light by pure steroids in ethanolic solution. Samples containing 2.5-20 µg of corticosterone or cortisol in 5 ml absolute ethanol were prepared and their absorption measured in Quartz cells of 4.5 ml capacity in the Unicam spectrophotometer using a hydrogen lamp as light source. The absorption peaks of both compounds was found to be at 242 mµ. At this wavelength the readings followed Beer's Law (see Fig.VI). It was however, not possible to use this simpler technique instead of the BT reaction because the paper blanks were high and irregular. When dried eluates of four different paper blanks (25 cm^2) from two different papers were taken up in 5 ml ethanol, readings of 0.080, 0.078, 0.095 and 0.057 were obtained at 242 mµ. These figures increased rapidly when read at a lower wavelength. They did not show very much change between 242 and 270 mµ. The eluates of two paper strips, each of them containing 10 µg corticosterone, gave readings of 0.150 and 0.194 at 242 mµ read against ethanol. When read against the different paper blanks the recoveries ranged from 57 to 148%. Fig.VII shows an example of such an experiment.

Figure VII



Absorption of ultra violet light by corticosterone eluted from paper.

A: 10 μg corticosterone, eluted from paper (36 cm^2).
B: Paper blank (36 cm^2)
C: 20 μg pure corticosterone
D: 10 μg pure corticosterone
---- A read against B
Abscissa: wave length;
Ordinate: extinction.

VI. Recovery Experiments

Quantities of 10 µg cortisol and corticosterone were added to arterial plasma and subjected to the extraction, separation, elution and estimation procedure with BT, outlined above. The recoveries averaged 70%. When 10 µg quantities of pure corticosteroids were put on paper, 80 to 90% could be recovered by the elution process. Exposure of the corticosteroid spots on paper to ultra violet light emitted by the scanner for a period of 2 to 5 minutes did not cause any detectable destruction of the compounds. After elution, the amounts recovered ranged again between 80 and 90% of the total amount present. Elution with a fourth portion of ethanol, or shaking for longer than 5 minutes tended to increase only the paper blanks.

Once it had been established that rat adrenals do not produce any detectable amounts of cortisol, 10 µg cortisol were added to the plasma or the gland homogenates in all experiments on rats and thus an estimate of recovery was obtained for each single experiment. These figures are included in the tables of results. Since however, the different compounds were dealt with separately during the elution process, no correction for variation in recovery was made of the figures obtained for the nat-

urally occurring compound(s). Only in a few experiments, in which a losse occurred obviously before the chromatographic separation, were corrections made. A recovery within the normal range was taken as an indication for good reliability of the result of this experiment.

Paper blanks were taken from corticoid free paper regions where no extract had travelled. Eluates of apparently corticoid free regions of the paper where the extract had travelled, gave a somewhat stronger reaction with BT. When read against paper blanks, the readings corresponded in different experiments to 0.3 to 2 µg corticosterone. Because of the possible presence of small amounts of undetected α-ketols in adrenal or blood extracts, the utilization of extract free paper regions for blanks seemed however, more justifiable.

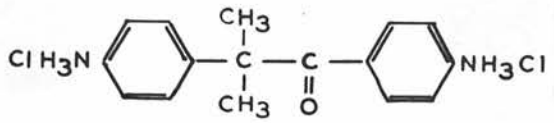
C. Histology

Adrenal glands were immersed for 24 hours in Orth's mixture, consisting of 1 part 40% formaldehyde and 9 parts of Mueller's fluid (2.5% $K_2Cr_2O_7$ and 1% Na_2SO_4). In this mixture, the catechol amines in the medulla get oxidised and form a brown pigment. The intensity of the reaction permits a rough estimate of their concentration. After washing in running water for 24 hours and embedding in 20% gelatine frozen sections of about 20 µ thick-

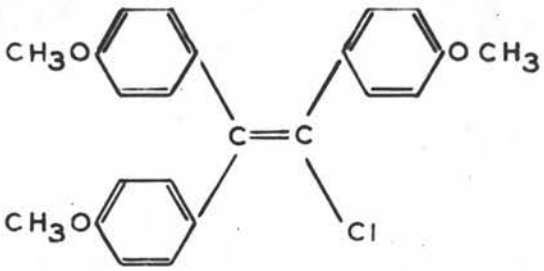
ness were cut. The sections were fixed in formalin vapour for 30 minutes, stained with Sudan IV and haematoxylin and covered with gum syrup. After this treatment the lipids appear as dark red droplets.

D. Evaluation of Results

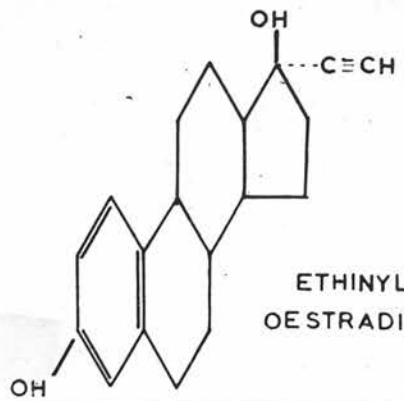
The standard errors of the means were calculated either from the squares of the deviations or from the range (Lord, 1947). The significance of the difference between the means of two groups was tested by the t-test. The values for P included in the tables, are given as < 0.1, < 0.05, > 0.05 and < 0.01. A difference was considered as "highly significant" when P was < 0.01; as "significant" when P was < 0.05.



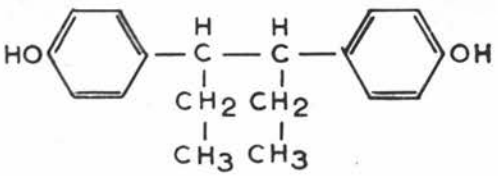
AMPHENONE B
1, 2-BIS-(P-AMINOPHENYL)-2-
METHYLPROPANONE-1; 2 HCl



TACE
(CHLOROTRIANISENE)
TRI-P-ANISYL CHLOROETHYLENE



ETHINYL
OESTRADIOL



HEXOESTROL

List of drugs

ACTH-Armour, 2.5 i.u./mg (Distributed by the Medical Research Council).

ACTH-Organon, soluble, 1.16 i.u./mg.

ACTH-Organon, long acting, Cortrophin 'Z'. (porcine origin) (through the courtesy of Dr. G. Dekanski).

Adrenaline base, Burroughs Wellcome.

Albumin, (bovine, 30% Armour, 20% Behring).

Amphenone 'B', 1,2-bis(p-aminophenyl)-2-methylpropanone-1. 2HCl. (Gift to Dr. M. Vogt from Dr. R. Hertz, National Cancer Institute, Bethesda).

Chloralose, British Drug Houses.

Chlorotrianisene; Tri-p-anisyl-chloroethylene, TACE. Wm. S. Merrell and Co., Cincinnati (through the courtesy of Dr. C.R. Thompson).

Cholesterol, recrystallised.

Ethinyloestradiol, (Gift from Ciba Laboratories to Dr. G.S. Boyd, Biochemistry Department, University of Edinburgh.

Erucic acid. (Gift from Dr. K.K. Carroll, Collip Medical Research Laboratories, University of Western Ontario, London).

Estradiol-17β. (Gift from Professor G.F. Marrian, F.R.S., Biochemistry Department, University of Edinburgh.)

Heparin B.P. 99-110 i.u./mg.

Hexoestrol, British Drug Houses.

Histamine acid phosphate, British Drug Houses.

Pentobarbitone, Nembutal Abbott Laboratories, Ltd. London.

Urethane B.P.

Progesterone, (Gift to Professor G.F. Marrian,F.R.S. from Chemical Specialities Co., Inc. New York).

List of Abbreviations

adr. - adrenal

b.p. - blood pressure

b. wt. - body weight

coll. time - collection time

expt. - experiment

gld - gland

hex. - hexoestrol

i.p. - intraperitoneally

i.u. - international units

i.v. - intravenously

l. adr. - left adrenal

ol. arach. - arachis oil

Progest. - progesterone

s.c. - subcutaneously

temp. - temperature

x - Bush's compound X

Kendall's alphabetical designations were used for the corticosteroids.

Figure VIII

SPECIES	ADRENAL GLAND					ADRENAL VEIN BLOOD				
	F	E	B	A	X	F	E	B	A	X
RAT	−	−	+++	−	+	−	−	+++	+−	+
GUINEA PIG	+++	−	−	−	−	+++	−	−	−	−
RABBIT	−	−	+	+++	+	−	−	+++	+	+
CAT	++	2.	++	+	+	++	−	++	+	+
DOG	+++	2.	+	−	−	+++	−	+	−	−
MONKEY	+++	−	+	−	−	+++	−	+	−	−

Corticosteroids found in adrenal gland extracts and adrenal vein blood of different species.

CHAPTER 3

Nature of Corticosteroids secreted and stored by adrenals of different species

Figure VIII gives a survey of the corticosteroids identified in adrenal vein blood and adrenal glands of different mammalian species by the methods outlined above. The following substances were found.

A. Rat

The main corticosteroid present in adrenal vein blood of rats, which gives the soda fluorescence and a positive reaction with BT, has the same R_F value as corticosterone. It is regularly accompanied by a substance which is somewhat more polar, absorbs ultra violet light of 240 mμ, and shows a primrose fluorescence in ultra violet light after treatment with strong alkali. It is present in almost 1/4 of the amount of corticosterone. Its structure is still unknown. It has also been observed by Reif and Longwell (1956) in adrenal vein blood of rats. As it is not always completely separated from the corticosterone spot, the two spots were always eluted together and the results expressed as corticosterone. In many samples, a third compound was present which is less polar than 11-dehydrocorticosterone and gives a positive soda fluorescence test. It is probably identical with Bush's compound X in rat

adrenal vein blood, which he provisionally identified as 11β-hydroxy-androstene-dione (Bush, 1953a). It has also been found in sheep adrenal vein blood (Bush and Ferguson, 1953). Neher and Wettstein (Wettstein, 1954) extracted considerable quantities of this compound from hog adrenals and identified it chemically and physically. In addition to these 3 compounds, occasionally a fourth one was visible, which has the same R_F-value as 11-dehydrocorticosterone and gives a positive reaction with BT and in the soda fluorescence test.

Chromatograms of extracts from rat adrenals show exactly the same steroid pattern. The relative size of the different spots is also similar to that in adrenal vein blood extracts.

Pretreatment of rats with substances which increase or decrease the secretion rate and the hormone stores influence all compounds to approximately the same degree. When the adrenal secretion was inhibited (for example, after treatment with oestrogens) there was never any indication that another steroid was produced which was taking the place of corticosterone.

When rats were fed with amphenone "B", several spots which absorb ultra violet light appeared on the chromatograms of the adrenal blood extracts. These spots did not give any other reaction typical

for Δ^4-unsaturated steroids or indicating an 4-ketol side chain. They were also present in extracts of arterial blood of these animals (Vogt, 1957), but could never be detected in gland extracts of amphenone treated rats.

B. Guinea pig

In one adrenal vein blood sample and one gland extract, mainly 17-hydroxycorticosterone was detected, accompanied by a trace of corticosterone. Some fluorescing spots of unknown nature were visible between the origin and the cortisol region. This observation was confirmed in many more animals by Fajer and Vogt (unpublished).

C. Rabbit

In rabbit adrenal vein blood corticosterone is the main C_{21} corticosteroid. In addition, small amounts of 11-dehydrocorticosterone and cortisone were found (Kass et al. 1954; Vogt, 1955). Again, the same compounds were detected in extracts of rabbit's adrenals. But whereas in adrenal blood extracts the ratio A/B was definitely less than one, it was more than one in the extracts of the twelve glands examined.

D. Cat

The adrenal vein blood of cats contains corticosterone and cortisol as major components. In

fourteen different samples from four different cats the ratio F/B varied between 0.95 and 3.5 (estimated in the eluate with BT). The mean was 1.56. In addition to these two compounds, a fluorescing spot in the region of 11-dehydrocorticosterone was clearly visible in ten samples after alkali treatment. In six samples a faint fluorescence in the region of Bush's compound X was present. The extracts of two cat adrenals showed definite fluorescing spots in the region of cortisone and corticosterone, and faint spots in the 11-dehydrocorticosterone, cortisone and X regions.

E. Dog

In dog adrenal vein blood extracts cortisol and corticosterone were found. The ratio F/B varied between 1.6 and 3.5 in four different samples from one dog. In an extract of the adrenal gland of a puppy spots in the region of cortisol and of corticosterone gave the soda fluorescence. The one in the cortisol region had about double the size and intensity of the one in the corticosterone region. There was a distinct, but small spot in the cortisone region.

F. Monkey

The two main steroids secreted by the adrenals of monkeys are also corticosterone and cortisol. In five different samples from one animal

the ratio F/B varied from 4.8 - 10.9. One extract of monkey adrenal was chromatographed. In the soda fluorescence test an intense spot was visible in the cortisol region and a smaller one in the corticosterone region.

Discussion

Results of investigations on the nature of the corticosteroids present in adrenal effluent of different species has been reported by Bush (1953); Farrell (1953); Zaffaroni and Burton (1953); Kass et al. (1954) and others. Our observations are in good agreement with the findings of these authors. Of the components present in major concentrations only corticosterone or cortisol have been identified. No cortisol was observed in the rat (Bush, 1953; Singer and Stack-Dunne, 1955) and in the normal rabbit (Bush, 1953; Kass et al. 1954). The figures reported for the ratio F/B vary, but all authors agree that in monkey, dog and cat cortisol is present in larger amounts than corticosterone.

It has already been pointed out that the brief collection periods (4-20 min) employed are not suitable for the detection of the small amounts of aldosterone present in adrenal vein blood. In rats, for example, hardly more than 0.1 µg aldosterone

can be expected to be present in an adrenal vein blood sample collected for 30 minutes from a 330 g rat (calculated from the data of Singer and Stack-Dunne, 1955).

No explanation is yet available for the considerable differences between species. There is no indication that the main steroid, which predominates in a given species, has the highest biological activity, when administered in pure form to members of that species.

No evidence was obtained that adrenal glands store significant amounts of any C_{21}-steroid, which is not also present in the adrenal effluent. With the exception of the rabbit, the ratio between the different compounds in adrenal vein blood and in adrenal glands of the same species appears to be similar. None of those C_{21}-steroids (for example, pregnenolone, progesterone, desoxycorticosterone, etc.) which can be transformed into corticosterone or cortisol when perfused through adrenals in vitro (Hechter et al. 1951), was detected in these experiments. If they are present at all in adrenals of these species then they are present in much smaller quantities than the actual secretion products. The amounts of the preformed secretory products of the adrenal cortex found in gland extracts are very

small considering the high rate of secretion (Vogt, 1943). For example, the amount of corticosterone present in a rat adrenal is equivalent to the amount secreted in 3 minutes (see Chapter 8). The rate of synthesis must therefore be very high. The absence of detectable amounts of C_{21}-intermediates in adrenal tissue suggests that the synthesis from earlier precursors (presumably cholesterol) takes place very rapidly.

No qualitative changes in the steroid pattern of rat adrenal extracts takes place after treating the animals with ACTH, hexoestrol, amphenone "B", adrenaline, histamine or after a severe haemorrhage. The small amounts of aldosterone presumably present in these adrenals, were not detectable by the method employed. The same holds for the large number of other trace-steroids reported to occur in extracts of adrenal glands. Whether these latter compounds have any functional significance is in any case, questionable (see Hechter and Pincus, 1954).

Apparently the biological activity of extracts from adrenal vein blood or adrenal glands can be fully accounted for by the observed corticosteroids plus aldosterone.

Adrenocortical Secretion of Normal Rats

The quantity of corticosterone produced by the left adrenal of adult male rats under conditions of operative stress has been measured in forty-three animals. The results are listed in Tables 3, 4, 5 and 6. The experiments were carried out during a period of two years, on rats of different origin with different anaesthetics. They are divided into four groups. Group 1 includes all experiments done under urethane anaesthesia in the winter of 1954-1955. Groups 2 and 3 contain the animals from which blood was collected under pentobarbitone anaesthesia. Group 2 consists of B-rats used from March, 1955 to March, 1956; group 3 of T-rats, used from March, 1956 to October, 1956. The fourth group consists of B-rats operated on under urethane anaesthesia in October, 1956.

The mean weights of the left adrenals expressed in mg/kg body weight were the same in all four groups. The mean figures for the amount of corticosterone secreted by the four groups ranged from 25.1 to 33.1 µg/g adrenal/minute and from 95 to 135 µg/hr/adrenal/kg body weight. If one takes into account a loss of about 30% during the process of extraction and elution, these figures fall within the range found by Singer and Stack-Dunne (1955)

TABLE 3

"CONTROL GROUP 1"

Cortical secretion of the left adrenal of untreated control rats in urethane anaesthesia (November, 1954 to January, 1955).

Date	Rat No.	Strain	b.wt. (g)	Coll. time (min)	plasma (g)	Mean body temp. (°C)	L.adrenal mg/kg b.wt.	CORTICOSTERONE SECRETION		% recovery	NaOH fluorescence
								µg/g adr./min	µg/hr/adr./kg b.wt.		
16.10 1954	3	E	398	60	5.4	37	52.8	20.8	65.6	-	x:-
22.10 1954	7	E	400	60	3.1	38.5	53.5	20.1	64.5	-	x:-
9.11 1954	13	T	360	25	1.2	36.5	68.0	32.3	132.0	-	-
26.11 1954	22	O	270	25	1.2	38.0	65.2	19.1	74.7	71	-
6.12 1954	27	B	340	40	-	37.5	55.6	27.5	81.9	88	x:-
15.12 1954	34	O	287	30	2.5	38.5	60.8	31.5	114.0	63	x:+

Contd:

TABLE 3 - CONTD

Date	Rat No.	Strain	b.wt. (g)	Coll. time (min)	plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b.wt.	CORTICOSTERONE SECRETION		% recovery	NaOH fluorescence
								µg/g adn/min.	µg/hr/adn/kg b.wt.		
11.1 1955	39	O	320	20	1.1	36	71.9	22.1	95.2	73	x:-
11.1 1955	40	B	310	20	1.5	38.5	62.0	31.2	116.0	73	x:-
19.1 1955	45	B	380	30	2.5	36.5	55.3	17.8	59.0	79	x:?
20.1 1955	48	O	260	20	1.7	34.5	78.2	34.0	159.0	74	x:-
25.1 1955	53	B	330	20	1.0	38	65.2	22.8	89.3	88	-
					mean ± S.E.		62.6 ± 2.4	25.4 ± 1.5	95.6 ± 9.5		

TABLE 4

"CONTROL GROUP 2"

Corticol secretion of the left adrenal of untreated control rats (B-strain) in pentobarbitone anaesthesia (March, 1955 to March, 1956).

Date	Rat No.	b. wt. (g)	Coll. time (min)	plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b. wt.	CORTICOSTERONE SECRETION		% recovery	NaOH fluorescence
							µg/g adr./min.	µg/hr/adr./kg b. wt.		
14.3 1955	73	325	15	2.5	37	63	33.3	127.0	62	x:++ A:?
22.3 1955	74	350	18	1.4	34.5	64	17.9	68.5	59	x:-
22.3 1955	75	370	18	1.4	-	62	24.0	88.5	75	x: ?
25.3 1955	76	345	16	0.72	-	68	19.5	80.5	93	x:-
25.3 1955	77	370	17	1.6	35.5	52	20.1	63.0	72	x:+
28.3 1955	78	370	15	1.8	-	54	25.0	81.0	67	x:+++
30.3 1955	80	310	15	1.6	-	72	15.6	67.1	65	x:++

Contd:

TABLE 4 - CONTD

Date	Rat No.	b. wt. (g)	Coll. time (min)	plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b. wt.	CORTICOSTERONE SECRETION		% recovery	NaOH fluorescence
							µg/g adr./min.	µg/hr/adr./kg b. wt.		
11.4 1955	81	370	15	2.5	-	53	32.3	105.0	75	x:+
11.4 1955	82	350	15	1.9	-	82	18.7	91.5	72	x:+
16.2 1956	165	262	15	2.4	34	79	24.3	115.0	72	x:+
16.2 1956	166	325	15	1.15	38	61	32.0	117.0	66	x:+
16.2 1956	167	318	15	2.3	37.5	67	26.4	106.0	76	x:?
16.2 1956	168	330	15	1.95	39.5	57	37.2	127.0	73	x:+
				mean ± S.E.		64 ± 2.5	25.1 ± 1.8	95.1 ± 5.0		

TABLE 5

"CONTROL GROUP 3"

Cortical secretion of the left adrenal of untreated control rats (T-strain) in pentobarbitone anaesthesia (June to October, 1956).

Date	Rat No.	b. wt. (g)	Coll. time (min)	plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b.wt.	CORTICOSTERONE SECRETION		% recovery	NaOH fluorescence
							µg/g adr/min	µg/hr/adr/kg b. wt.		
16.7 1956	273	286	15	1.2	39.5	79	42.2	215	63	x:++
6.9 1956	282	345	15	1.9	39	67	29.2	117	84	x:++ A:?
6.9 1956	283	314	15	3.3	36	56	41.1	137	-	x:++
10.9 1956	284	338	15	2.3	33	74	26.7	118	75	x off paper
10.9 1956	285	318	15	1.6	37	69	39.5	162	72	x:++
10.9 1956	286	374	15	2.5	36	43	31.3	81.3	76	x:?
13.9 1956	287	327	15	1.4	35	74	21.5	95.5	78	x:off paper

Contd:

TABLE 5 - CONTD.

Date	Rat No.	b.wt. (g)	Coll. time (min)	plasma (g)	Mean body temp. (°C)	L. adrenal mg/kg b.wt.	CORTICOSTERONE SECRETION		% recovery	NaOH fluorescence
							µg/g adr./min	µg/hr/adr./kg b. wt.		
13.9 1956	288	373	15	1.4	39.5	64	40.8	155	63	x:++
13.9 1956	289	310	15	1.1	38	74	38.0	169	69	x:++
18.9 1956	290	293	15	1.4	37	57	34.5	119	69	x:+
18.9 1956	291	242	15	2.2	35	80	22.1	106	65	x:+
18.9 1956	292	294	15	1.5	38	71	33.2	142	70	x:+
18.9 1956	293	280	15	1.7	36	73	30.5	134	72	x:++
				mean ± S.E.		67.7 ± 3.1	33.1 ± 1.7	135 ± 10.3		

TABLE 6

"CONTROL GROUP 4"

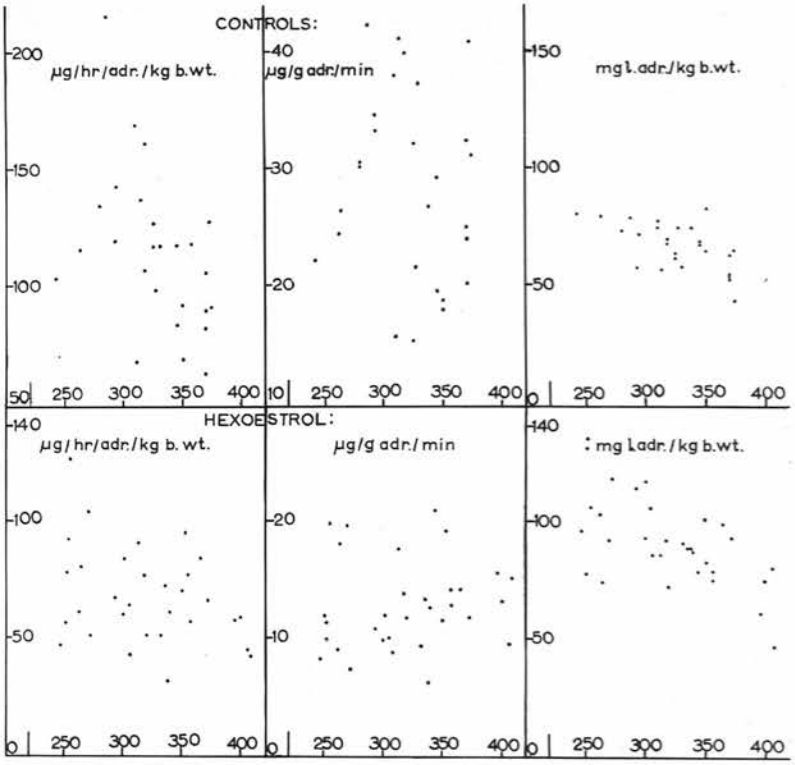
Cortical secretion of the left adrenal of untreated control rats (B-strain) in urethane anaesthesia (October, 1956).

Date	Rat No.	b. wt. (g)	Coll. time (min)	plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b.wt.	CORTICOSTERONE SECRETION µg/g adr/min	CORTICOSTERONE SECRETION µg/hr/adr/kg b. wt.	% recovery	NaOH fluorescence
1.10 1956	302	324	15	1.4	36.5	66.2	29.6	117	70	x:++
1.10 1956	303	328	"	1.2	37.5	60.4	29.6	107	76	x:++
1.10 1956	304	303	"	0.85	-	48.2	33.8	98	65	x:+
1.10 1956	305	330	"	1.5	38.5	53.1	27.1	86	73	x:+
1.10 1956	306	306	"	1.2	38	61.0	29.5	108	62	x:++
1.10 1956	307	307	"	1.0	39	64.0	27.8	107	58	x:++
				mean ± S.E.		58.8 ± 2.9	29.56 ± 1.08	103.8±5		

and by Reif and Longwell (1956). There is no significant difference between the means of group 1 and 2, nor between the means of group 3 and 4. The means of groups 3 and 4 are however, significantly higher than those of groups 1 and 2. Therefore, in the experiments to test the influence of drugs on the secretory capacity of the rat adrenal, care was taken to ensure that the controls were from the same stock as the test animals, and were operated on under the same anaesthetic and approximately the same date.

In addition to the data on secretion rate and adrenal weight, the tables include body weight, amount of plasma collected over a given period of time, mean body temperature during blood collection, percentage recovery of cortisol added to each plasma sample before the extraction procedure, and the results of the soda fluorescence test, carried out on the remainders of the paper chromatograms after the cortisol and corticosterone regions had been cut out. In the section "NaOH-fluorescence", +, ++ and +++ indicate increasing intensities and minus the absence of detectable fluorescence. The amounts of plasma yielded were used as an indication of the quantity of blood circulating through the gland. Where the flow was so slow that the sample yielded less than one g plasma/20 minutes the data were discarded. The amount of blood collected is however,

Figure IX



Body weight plotted against:

1. Corticosterone secretion (µg/hr/adr./kg b.wt.)
2. " " (µg/g adr./min.)
3. Weight of left adrenal (per kg b. wt.)

Abscissa: body weight (g); ordinate: as indicated in the Figure.

not an absolute measure of the blood flow through the adrenal, because small tributaries to the adrenal vein, which do not come from the gland are always present but were not tied, in order to avoid handling the tissue near the adrenal.

From the upper diagrams of Figure IX it can be seen that in adult rats there is no correlation between body weight and secretion rate.

CHAPTER 5

Effect of ACTH on adrenocortical secretion of the intact rat

The experiments described in this section were devised in order to determine the effect of exogenous ACTH on the function of the adrenal cortex of rats with intact pituitary glands. ACTH was administered in three different ways. In the form of: 1. an intravenous infusion before and during collection of an adrenal blood sample; 2. subcutaneous injections of large quantities within a short period of time; 3. subcutaneous injections of smaller quantities over a longer period of time.

1. Collection of adrenal vein blood from a rat is at present possible only after laparotomy under anaesthesia. This procedure involves the release of ACTH from the anterior pituitary. Therefore the amount of corticosterone found in adrenal vein blood of rats provides only information on the secretory capacity of the gland when exposed to increased amounts of circulating ACTH. It was of interest to examine whether, during the stress of this operation, exogenous ACTH administered in the form of a slow intravenous infusion is able to increase further the output of corticosterone, or whether

such an adrenal is already secreting at its maximal capacity.

2. The second group of experiments was carried out in order to examine the secretory capacity of an adrenal which was intensely stimulated and became acutely enlarged during the 36 hour period preceding adrenal blood collection.

3. In the third group of experiments, chronic adrenal hypertrophy was induced by a number of daily injections of long acting Cortrophin'Z' and the secretory capacity of such glands measured under conditions of the usual operative stress.

Methods

1. Intravenous infusion

Experiments were carried out on 10 normal male rats (Table 7). In all instances, blood was collected under pentobarbitone anaesthesia. For details of the operative procedure see p. 9). A control sample (S_1) was collected over 15 minutes. At the end of this period collection was interrupted, adrenal outflow directed back into the animal and infusion started. 0.25, 0.28 or 0.31 i.u. ACTH (Armour) dissolved in 2 ml saline were infused into the right femoral vein at a speed of 0.25 ml/min. Collection of a second blood sample (S_2)

was commenced at the end of the fourth or the sixth minute of the infusion period and was continued for 15 minutes. In order to maintain the blood pressure constant, repeated injections of normal rat blood were given (details see p 10). The amounts necessary ranged between 1 and 10 ml in the different animals.

A control experiment was carried out on 3 rats (Table 8). Two consecutive adrenal blood samples were collected from each of them, but no ACTH infused. Blood pressure was maintained constant by 5 to 10 injections of 0.5 ml blood.

2. Short term pretreatment with large doses of ACTH

Four groups of rats were injected with 16 to 64 i.u. ACTH during the 36 hour period preceding blood collection (Table 9). Adrenal vein blood was collected in urethane anaesthesia 1 to 3 hours after the last injection. Injections were given according to the following plan:

Time:	long acting ACTH+ (i.u.):				soluble ACTH++ (i.u.):			
1st day: 9 p.m.	5	5	5	1	4	4	4	2
2nd day: 9 a.m.	5	5	5	1	4	4	4	2
1 p.m.			5		4	4	4	2
5 p.m.			5		4	4	4	2
9 p.m.	5	5	5	1	4	4	4	2
3rd day: 9 a.m.	5	5	5	1	4	4	4	2
Group:	I	II	III	IV	I	II	III	IV

+Cortrophin 'Z', long acting corticotrophin (Organon)

++ACTH Armour, soluble.

Control group 1 (Table 3), served as controls for group I, control group 4 (Table 6), for groups II and III.

3. Long term pretreatment with small doses of ACTH

Five rats of different origin received 11 to 14 daily injections of 1 i.u. long acting Cortrophin'Z' (Organon) (henceforth called ACTH'Z'). 18 to 24 hours after the last injection adrenal vein blood was collected under urethane anaesthesia (Table 10). Eleven untreated rats of the same strain served as controls. They were also operated on under urethane anaesthesia (control group 1, Table 3).

A second group of 4 rats (E- and T-strain) was pretreated with 4 daily subcutaneous injections

of 1 i.u. ACTH'Z', and a third group of 3 rats (E-strain) with a combination of 4 daily subcutaneous injections of 1 i.u. ACTH'Z' per rat and 0.8 ml arachis oil/kg b. wt. On the fifth day, adrenal vein blood was collected under pentobarbitone anaesthesia (Table 10). The mean secretion rate and adrenal weight of the 26 untreated rats of control groups 2 and 3 (Tables 4 and 5) were used for comparison.

Results

1. Intravenous infusion

The effect of an intravenous infusion of ACTH in normal rats during adrenal blood collection under anaesthesia was very small. An increase in corticosterone output during and after infusion, was only observed in rats whose corticosterone secretion during the collection of the control sample was, for unknown reasons, below the normal mean. This increase ranged between 30 and 53% and was accompanied by an increase in the secretion of Bush's compound X (group 1, Table 7). Rats with normal or high initial secretion showed no further rise as a result of the infusion of ACTH (group 2, Table 7). ACTH caused a marked increase of

TABLE 7

ACTH-infusions

The effect of an i.v. infusion of ACTH into normal rats on the amount of corticosterone secreted by the left adrenal. A first blood sample (S_1) was collected for 15 min. and ACTH infused for the next 8 min. Sample 2 (S_2) was collected during the last 4 - 6 min. of the infusion and the following 11 - 9 min. All blood collections were done under

Group No.	Date	Rat No.	b.wt. (g)	Infusion		mean b. temp. (°C) S_1	mean b. temp. (°C) S_2	mean b.p. S_1	mean b.p. S_2	plasma (g) S_1	plasma (g) S_2
1	23.2 1956	77	370	0.25u ACTH in 2 ml saline infused during 8 min.		35.5	35	85	55	1.5	1.5
	28.3 1956	78	370			-	-	105	85	1.8	2.3
	30.3 1956	80	310			-	-	110	100	1.6	3
	11.4 1956	82	350			-	-	125	100	1.9	4.3
	13.9 1956	287	327			34.5	35.5	90	75	1.4	2.8
			mean (1 - 5) ± S.E.:								
			significance of difference between S_1 and S_2:								
2	11.4 1956	81	370	Infusion of 2 ml saline during 8 min. containing i.u. ACTH:	0.25	-	-	100	84	2.2	4.3
	6.9 1956	282	345		0.31	39	39	160	130	1.9	5.0
	5.9 1956	283	314		0.28	36	36	120	75	3.3	5.5
	13.9 1956	288	373		0.25	39.5	39.5	115	115	1.4	2.4
	13.9 1956	289	310		0.25	38	38	100	95	1.1	2.8
			mean (6 - 10) ± S.E.:								
			mean (1 - 10) ± S.E.:								

TABLE 7 Contd

pentobarbitone anaesthesia. Corticosterone secretion during the control period was lower than the normal mean of 29.1 µg/g adr./min. in Group 1, and higher in Group 2.

Rat No.	% recovery		NaOH-fluorescence		B-secretion: µg/g adr./min.		% change
	S_1	S_2	S_1	S_2	S_1	S_2	
77	72	88	x:+	x:++	20.1	30.7	+52.8
78	67	72	x:+++	x:+++	25.0	42.5	+70.0
80	65	65	x:+	x:++	15.6	20.3	+30.1
82	72	82	x:+	x:++	18.7	25.6	+36.9
287	78	85	-	x:++	21.5	32.9	+53.1
					20.2 ± 1.8	30.4 ± 4.3	+48.7
					P = 0.05-0.02		
81	75	88	x:+	x:+++	32.3	33.2	+3.0
282	84	83	x:+	x:+	29.2	25.5	-12.7
283	-	-	x:+	x:+	41.1	39.6	-3.6
288	63	81	x:++	x:++	38.3	41.9	+9.4
289	69	99	x:++	x:++	38.0	43.5	+14.5
					35.8 ± 2.3	36.7 ± 3.5	
					28.1 ± 2.6	33.6 ± 2.4	

TABLE 8

Corticosterone content of two consecutive samples of adrenal vein blood collected from the same rat for 15 minutes under pentobarbitone anaesthesia.

Date	Rat No.	b. wt. (g)	mean b. temp. (°C)		mean b.p. (mm Hg)		plasma (g)		% recovery		B-secretion: µg/g adr./min		Change (%)
			S_1	S_2	S_1	S_2	S_1	S_2	S_1	S_2	S_1	S_2	
18.9 1956	291	242	35	35	100	80	2.2	2.0	65	74	22.1	21.1	-4.5
18.9 1956	292	294	38.4	38	120	110	1.5	1.4	70	72	33.2	33.7	+1.5
18.9 1956	293	280	36	36	80	85	1.7	1.7	72	68	30.5	32.5	+6.7
					mean ± S.E.:						28.6 ± 3.8	29.1 ± 4.3	

TABLE 9

Short term pretreatment with ACTH

Effect of large doses of ACTH (24 - 46 i.u./rat, administered subcu-

Group	Date of blood coll.	Rat No.	Strain	Treatment	b.wt.(g) at start	b.wt.(g) at end	(min.) Coll. time	plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b.wt.
	(n=11)			Controls under urethane: Group 1 (see Table 3)	mean ± S.E.:					62.6 ± 2.4
I	16.2 1955	65	B	24 i.u. soluble and 20 i.u. long acting ACTH'Z'/rat	380	375	30	1.5	36.7	98
	16.2 1955	66	B		370	370	30	1.9	38.5	108
	28.11 1955	124	B		353	335	15	1.6	36.5	107
	28.11 1955	125	B		348	323	15	1.4	39	94
	13.12 1955	130	B		352	326	15	1.5	33	108
	13.12 1955	131	B		315	279	15	1.2	36	113
				mean ± S.E.:						105 ± 3
	Significance of difference from controls:									P < 0.01
	(n=6)			Controls under urethane: Group 4 (see Table 6)	mean ± S.E.:					58.8 ± 2.9
II	27.9 1956	299	B	24 i.u. soluble and 20 i.u. long acting ACTH'Z'/rat	348	325	15	1.7	37	106
	27.9 1956	300	B		327	320	15	3.0	37	97
	22.10 1956	335	B		286	274	15	1.9	37.5	126
	22.10 1956	336	B		321	316	15	1.3	38.5	105
	22.10 1956	337	B		347	336	15	1.5	38.5	112
III	23.11 1956	343	B	24 i.u. soluble and 40 i.u. long acting ACTH'Z'/rat	267	275	15	1.4	37	130
	23.11 1956	344	B		250	263	15	1.25	39	141
				mean ± S.E.:						116.7 ± 6.1
	Significance of difference from controls:									P < 0.01
IV	15.1 1955	43	0	12 i.u. soluble and 4 i.u long acting ACTH'Z'/rat	325	320	30	2.8	36	73
	15.1 1955	44	0		318	313	30	2.1	38.5	79

TABLE 9 Contd:
taneously in 6 doses during 36 hr) on the corticosterone secretion of the left adrenal. Blood collected under urethane anaesthesia over a 15 min. period, 1 to 3 hr after the last ACTH injection.

Rat No.	Corticosterone secretion		% re-covery	NaOH-fluor-escence	Histology
	µg/g adr./min	µg/hr/adr./kg b.wt.			
	25.4 ± 1.5	95.6 ± 9.5			
65	14.4	85	42	x:-	-
66	10.5	66	-	x:+	-
124	24.4	156	68	x:++	-
125	14.5	81	62	x:++	-
130	18.9	124	58	x:++	-
131	18.2	123	68	x:++	-
	16.8 ± 2.2	106.1 ± 14.5			
	P < 0.01	P > 0.1			
	29.6 ± 1.1	103.8 ± 5			
299	22.1	141	80	x:?	some lipid depletion in fasciculata
300	23.1	135	76	x:?	do. and glomerulosa
335	19.5	148	76	x:+++	some lipid depletion in fasciculata
336	27.6	174	69	x:+	some lipid depletion in fasciculata
337	18.5	124	75	x:-	some lipid depletion in fasciculata
343	17.9	139.5	68	x:++	normal
344	22.3	188.5	70	x:++	normal
	21.57 ± 1.35	150 ± 9			
	P < 0.01	P < 0.01			
43	20.1	88.2	79	-	some lipid depletion in fasciculata
44	20.0	96.2	79	-	some lipid depletion in fasciculata

Figure X

Corticosterone secretion of the left adrenal of rats, pretreated for a short period with large doses of ACTH.

adrenal blood flow in all rats, except No. 77. The increase ranged between 28 and 163%.

The corticosterone content of two consecutive adrenal blood samples, collected without infusion of ACTH was equal in the 3 examples tested, whether the secretion rate was high or low (Table 8).

2. Short term pretreatment with large doses of ACTH

The results are summarised in Fig. X.

As a result of the injection of very large doses of ACTH within a short period of time the adrenals became very enlarged and the corticosterone secretion per g adrenal decreased significantly (Table 9). Adrenal hypertrophy in the animals of group 1 amounting to 68%; corticosterone secretion per kg b. wt. was normal. In the rats of groups II and III, which were used approximately one year later, hypertrophy was more pronounced. In these animals the corticosterone secretion per kg b. wt. was about 50% higher than in the normal controls, although the secretion per g adrenal was decreased by about 30%. The lipid content of the glands showed in general, a tendency towards diminution.

3. Long term pretreatment with small doses of ACTH

Treatment with 11 - 14 daily injections of 1 i.u. ACTH'Z' was without effect on the amount of

TABLE 10

Long term pretreatment with ACTH

Corticosterone secreted by the left adrenal of rats pretreated with daily injections of long acting ACTH-Z for 11 - 14 or for 4 days. Blood collected approximately 24 hours after the last ACTH-Z injection

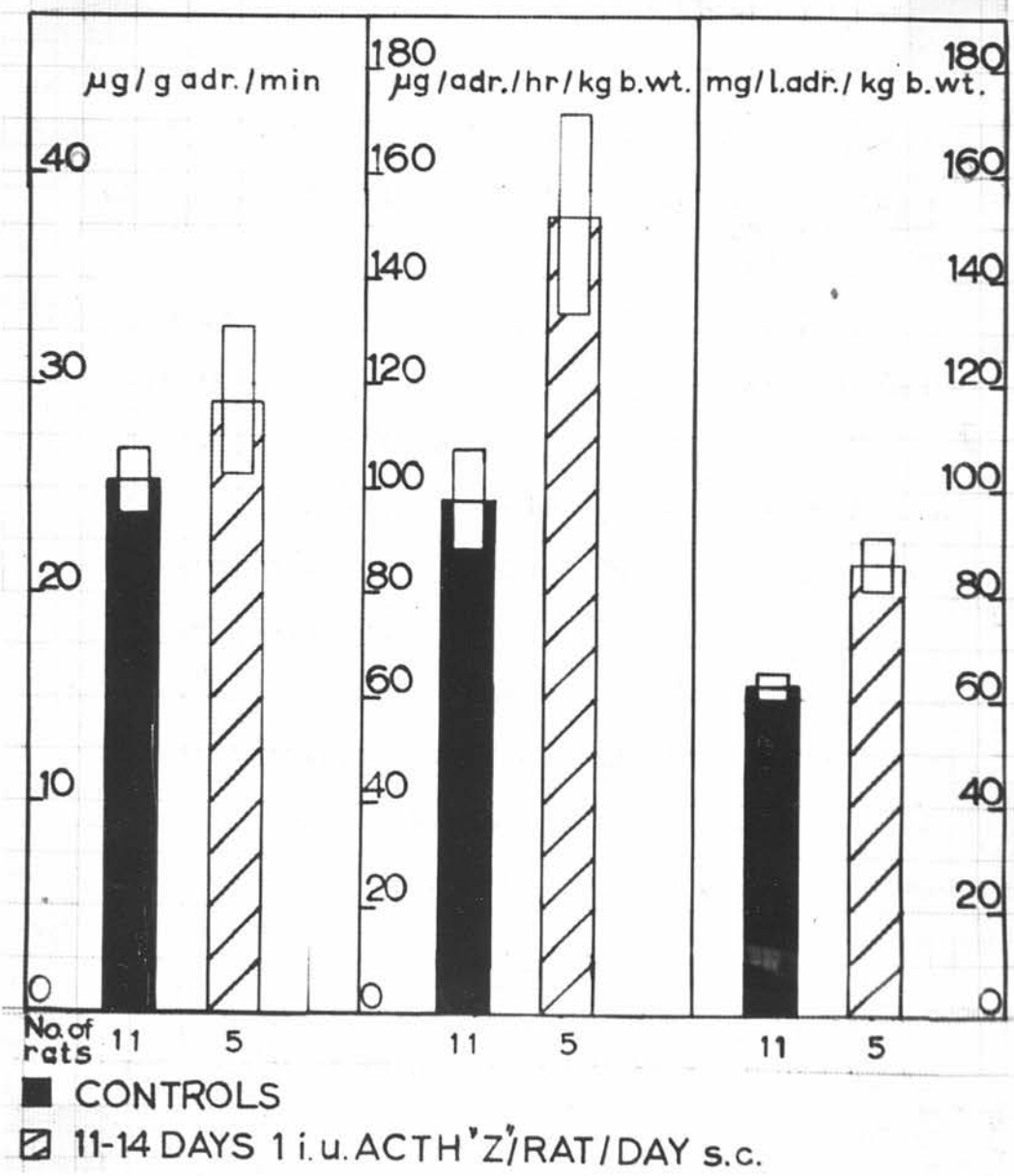
Blood coll. Date	Rat No.	Strain	Treatment	No. of inj.	b.wt. (g) at start	b.wt. (g) at end	Coll. time (min)	plasma (g)	Mean body temp. (°C)	l-adrenal mg/kg b.wt.
(n=11)			Controls in urethane: Group 1, see Table 3			mean ± S.E.:				62.6±2.4
17.12 1954	38	O	1 i.u. long acting ACTH Z per rat/day s.c.	11	310	305	20	1.0	38	89
1.2 1955	56	B		12	310	300	30	2.3	36.5	102
3.2 1955	59	B		14	328	316	30	1.7	39	84
3.2 1955	60	B		13	354	341	20	1.2	37	78
15.12 1955	133	E		14	300	310	15	1.0	38	79
			mean ± S.E.:							86±5
			Significance of difference from controls:							P < 0.01
(n=26)			Controls in pentobarb. Group 2 + 3, see Tables 4.5			mean ± S.E.:				66±2
11.6 1956	236	E	1 i.u. long acting ACTH Z/ rat + 0.8 ml ol.arach./kg b.wt./ day.s.c. for 4days		360	365	15	2.3	36	86
11.6 1956	237	E			315	316	15	2.1	37	97
11.6 1956	238	E			303	310	15	2.1	35	97
18.6 1956	247	E	1 i.u. long acting ACTH Z/rat/day for 4 days.		279	280	15	1.2	37.5	79
18.6 1956	248	E			292	290	15	1.6	38.5	79
2.7 1956	267	T			265	260	15	1.7	35.5	113
2.7 1956	268	T			265	263	15	-	38.5	65
			mean ± S.E.:							88±7
			Significance of difference from controls:							P < 0.0

TABLE 10 Contd:

under urethane (11 - 14 day group) or under pentobarbitone (4 day group) over a 15 min. period.

Rat No.	Corticosterone secretion µg/g adr./min.	Corticosterone secretion µg/hr/adr./kg b.wt.	% recovery	NaOH fluorescence	Histology
	25.4 ± 1.5	95.6 ± 9.5			
38	35.2	187	75	x:+++ A:+	All layers lipid laden.
56	23.8	146	79	x:+	All layers lipid laden.
59	41.2	207	96	x:+	All layers lipid laden.
60	23.0	110	96	x:-	glomerulosa and fasciculata lipid laden.
133	22.9	108	69	x:++	-
	29.2 ± 3.5	152 ± 19			
	P > 0.1	P=0.02-0.01			
	29.1 ± 1.6	115 ± 7			
236	26.7	137	81	x:++	All layers lipid laden.
237	33.4	187	60	x:++	All layers lipid laden.
238	22.3	129	74	x:+	All layers lipid laden.
247	26.7	126	42	-	All layers lipid laden.
248	26.0	128	78	-	All layers lipid laden.
267	19.7	134	86	x:?	normal
268	43.1	172	69	x:?	normal
	28.3 ± 3.3	145 ± 8.5			
	P > 0.1	P=0.05-0.02			

Figure XI



Corticosterone secretion of the left adrenal of rats pretreated for a long period with small doses of ACTH.

corticosterone secreted per g adrenal under conditions of operative stress. Since the adrenals were enlarged, the amount of corticosterone secreted per kg b. wt. was increased (see Fig.XI and Table 10). The hypertrophied adrenal cortices contained larger amounts of sudanophilic lipids than normal rat adrenals.

Similar structural and functional changes were observed after 4 daily injections of ACTH'Z'.

The possible influence exerted by fatty acids on corticosterone synthesis, suggested by their effect on adrenal cholesterol concentration, is discussed in Chapter 8. In the present experiments the possibility that combined treatment with ACTH and arachis oil could significantly increase corticosterone secretion per g adrenal tissue was examined. No increase was found.

Discussion

Under all conditions examined the influence exerted by exogenous ACTH on the secretory performance of the rat adrenal cortex was small. In the intact rat infusion of 250 to 310 m.u. ACTH did not elicit increased corticosterone secretion during collection of adrenal blood samples, unless the secretory performance of the gland had been be-

low the normal mean before the start of infusion. The quantities infused were sufficient to increase the amount of ACTH circulating under such conditions (up to 8 m.u./100 ml., Sydnor and Sayers, 1954) several fold. The presence in the ACTH preparation of significant amounts of vasopressin which could impair adrenal blood circulation and hence adrenal function, can be excluded as the cause of its lack of effect. During ACTH infusion, no rise in systemic blood pressure could be observed and adrenal blood flow consistantly increased. The negative result must thus be taken as an indication that in the rat, the amount of endogenous ACTH normally released in the course of laparotomy under anaesthesia is sufficient maximally to stimulate synthesis and release of corticosterone. A similar observation was made by Hechter et al. (1955) in the intact dog. No increase in the secretion of cortisol or corticosterone was found after ACTH infusion under conditions of operative stress. When adrenal vein blood was collected from a conscious dog by the method of Hume and Nelson (1954), intravenous infusion of ACTH increased the output of 17-hydroxysteroids from 2.8 ± 6 µg to 15.2 ± 1.3µg/min./adrenal. The latter figure lies within the

range of the secretion measured immediately after the insertion of the cannula under ether anaesthesia (Nelson et al. 1956). No satisfactory explanation is at present available for the stimulatory effect of an ACTH infusion on rat adrenals, which secrete below the normal mean. Impaired activity of the mechanism of release of endogenous ACTH in such animals might be one of the possible causes for a lower corticosterone secretion before infusion.

When rats received large amounts of ACTH during the 36 hour period preceding blood collection, the weight of the adrenals became nearly doubled. The secretory capacity per unit adrenal tissue was however, significantly decreased. This indicates that it is not only hormone producing tissue which accounts for the acute increase in adrenal mass. A proportion of it is obviously due to increase of blood volume and interstitial fluid. It seems also, that the enzyme systems necessary for the hormone synthesis are not immediately produced in the newly formed cells.

Under conditions of operative stress, the secretory capacity of adrenals enlarged and continuously stimulated by 4 to 14 daily injections of 1 i.u. ACTH'Z' was found to be within normal range.

Because of hypertrophy of the gland, the secretion rate per kg b. wt. was significantly increased. Daily administration of ACTH for over a week of porcine origin to rabbits led to the appearance of cortisol in adrenal vein blood, the ratio F/B being about 1:2. When treatment was continued for 3-4 weeks, the F/B ratio became 4 (Kass et al. 1954). In the rat, unlike in the rabbit, prolonged treatment with ACTH did not alter the pattern of cortical secretion.

Summary

The effect of exogenous ACTH on adrenocortical secretion was studied in intact rats under conditions of operative stress. Under these conditions intravenous infusion of ACTH only increased corticosterone secretion in rats which secreted less during operation than the normal mean. Short term pretreatment with large doses of ACTH resulted in a decreased secretion per g adrenal tissue, whereas the secretion per kg b. wt. remained either unchanged or even increased. Long term pretreatment with smaller doses of ACTH resulted in a normal secretion rate per g tissue and in an increased secretion rate per kg b. wt.

CHAPTER 6

Studies on the relationship between adrenal medulla and adrenocortical secretion

A functional inter-relationship between adrenal cortex and adrenal medulla was first suspected by Sjöstrand (1934), when he observed that in many instances increased adreno medullary secretion is accompanied by hyperemia of the adrenal cortex. Direct evidence for this supposition was first obtained by Vogt (1944). She observed a significant increase in the biological activity of adrenal vein blood when assayed by the Selye-Schenker test, in eviscerated splanchnotomized cats and dogs after intravenous infusion of adrenaline. She considered it likely that this effect of adrenaline was elicited by a direct action on the adrenal cortex.

A second manner in which adrenaline can influence adrenocortical activity is by stimulating the release of adrenocorticotrophic hormone. This has been deduced from the observation that adrenaline causes a fall in adrenal ascorbic acid and cholesterol concentration in intact, but not in hypophysectomised rats (Long and Fry, 1945). The existence of this mechanism was finally established by Farrell and McCann (1952) who found the blood level

of ACTH to be elevated after infusion of adrenaline into intact rats.

The effect of adrenaline on the anterior pituitary is not necessarily mediated by the nervous system. It could be elicited in rats whose pituitaries had been transplanted into the anterior chamber of the eye (McDernott et al. 1950) and also after high pontine section (Sayers, 1957). It has been suggested that the stimulation of the adrenal cortex which follows stress may be secondary to the release of adrenaline from the adrenal medulla (Long, 1947). But this is not the only mechanism involved. In rats adapted to the nonspecific stressing effects of the adrenaline antagonists SKF-501 and Dibenzyline, ACTH release following adrenaline injections could be prevented by these drugs. Under these conditions it was not possible to alter significantly the ACTH release induced by formalin or forced immobilization (Guillemin, 1955).

The following experiments were carried out in order to re-examine certain aspects of medullary cortical relationship by means of chemical micromethods now available for steroid estimations. The amount of corticosterone secreted into adrenal vein blood was measured in animals whose adrenals

adrenals were either demedullated or deprived of their nerve supply. The influence of adrenaline infusions on cortical secretion was measured under these conditions.

Methods

The experiments were carried out on 10 adult male rats, 4 cats, 1 dog and 2 monkeys (in collaboration with Dr. Mary Pickford). The pituitary glands of all animals were intact. Blood collections were invariably carried out by means of abdominal operations under anaesthesia.

1. Rats

In 8 adult male rats (Table 11) both adrenals had been enucleated (see p. //) one to six months prior to the adrenal blood collection. In 2 rats (No.338 and 340) the left adrenal had been enucleated and the right removed in toto $1\frac{1}{2}$ months prior to the collection of adrenal vein blood. From all rats a first sample (S_1) was collected over a 15 min. period. Then the adrenal vein blood was directed back into the animal and adrenaline infusion started. 5, 6.25 or 10 µg l-adrenaline base were dissolved in 4 ml saline and infused into the right femoral vein or the left

carotid artery at a speed of 0.5 ml per min. 4-6 min. before the end of the infusion the collection of a second 15 min. blood sample (S_2) was started.

2. Larger animals

Experiments were carried out on 4 cats, 1 dog and 2 monkeys. In cats and monkeys both adrenals were denervated in an aseptic operation 6 - 16 days before the experiment (see p. 12). In the dog, the splanchnic nerves were severed half-an-hour before the collection of the first adrenal blood sample. Dissection for cannulating the vein which drains adrenal blood is described on p. 12. From each animal, adrenal blood samples were collected for 1.5 to 8 min. (see Tables 12 - 18) before and during, or shortly after, infusion or injection of adrenaline by different routes. The exact data of each experiment are outlined in the Tables. Adrenaline was usually infused in the form of a 10^{-6} solution in saline at a speed of 10 µg/min.

Adrenal vein blood samples were centrifuged immediately after collection and the plasma was subjected to the purification and chemical estimation procedures described in Chapter 2.

Results

Only those corticosteroids were estimated which are secreted in major quantities. These were corticosterone in the rat and cortisol and corticosterone in cat, dog and monkey. The qualitative findings are discussed in Chapter 3. No information concerning the amounts of aldosterone secreted was obtained.

1. Rats

Details of the results obtained on the rats are given in Table 11. The mean corticosterone secretion per g tissue of a regenerated adrenal cortex after enucleation amounted to 30.8 ± 2.6 µg/min. and appears to be the same as that of a normal gland. Direct comparison is however, not possible. In the regenerates, the percentage of cortical tissue can be either higher than in normal glands because of lack of medullary tissue, or lower because of scar tissue formed. Since the regenerates were usually small the mean amount of corticosterone secreted per adrenal/hr/kg b. wt. was only 64.4 ± 5.6 µg. This figure is significantly lower than in the normal controls (compare Table 4 (95 µg ± 5 µg) and Table 5 (135 µg ± 10 µg). Again direct comparison is not possible because the

TABLE 11

Corticosterone secretion of demedullated rat adrenals; S_1 collected before, and S_2 during an infusion of adrenaline. With exception of rats No.338 and 340, in which the right adrenal was removed, both glands

Rat No.	Enucleation	Blood collection		Adrenaline infused:				S_2 started after min.	Mean b.p. (mm Hg)		Collect. time (min)		Plasma (g)	
		Date	b.wt. (g)	µg	route	in ml saline	speed (ml/min)		S_1	S_2	S_1	S_2	S_1	S_2
70	4.9 1954	10.3 1955	400	10	i.v.	1	0.06	1	-	-	17	17	1.3	1.1
93	3.5 1955	2.6 1955	390	5	i.v.	4	0.36	1	115	130	15	15	1.7	1.9
114	9.3 1955	19.7 1955	395	5	i.v.	4	0.4	1	100	100	15	15	1.9	1.6
116	15.4 1955	21.7 1955	445	6.25	i.v.	5	0.38	1	110	115	15	15	1.8	2.5
117	15.4 1955	21.7 1955	400	6.25	i.v.	5	0.38	1	92	100	20	20	3.3	3.7
118	16.4 1955	25.7 1955	450	5	i.v.	4	0.4	5	95	110	16	16	1.8	2.7
120	16.4 1955	27.7 1955	420	5	i. carot.	4	0.4	5	105	100	15	15	1.5	1.7
121	16.4 1955	27.7 1955	390	10	i. carot.	4	0.4	5	107	107	15	15	1.4	1.5
338	18.9 1956	29.10 1956	407	5	i.v.	4	0.5	4	-	-	15	15	1.4	1.6
340	18.9 1956	7.11 1956	434	5	i.v.	4	0.5	4	45	60	15	15	2.7	1.5

mean $\pm$ S.E.:

TABLE 11

had been emucleated.

Rat No.	Corticosterone secretion			1. adrenal (mg)	Recovery (%)		Change %
	µg/g adr./min.		µg/hr/adr./kg b. wt.				
	S_1	S_2	S_1		S_1	S_2	
70	27.3	28.1	93.5	22.8	55	55	+3
93	29.5	41.0	63.8	14.0	72	75	+39
114	27.3	35.7	70.0	16.8	64	65	+30
116	45.0	34.8	57.2	9.4	82	60	-22
117	34.6	33.2	54.0	10.4	82	85	-4
118	40.8	32.6	75.0	13.8	88	104	-20
120	39.3	35.1	74.3	13.2	98	69	-11
121	23.0	21.9	74.4	21.0	88	88	-5
338	20.0	20.5	37.4	12.7	69	84	+2.5
340	21.3	22.2	44.3	15.0	58	71	+4
	30.8 ± 2.6	30.5 ± 2.1	64.4 ± 5.6	14.9 ± 1.4			

TABLE 12

Cat 1 - male - 4.3 kg. Influence of adrenaline on the cortisol and corticosterone secretion of the denervated left adrenal (214 mg). Denervation 12 days prior to the experiment. Mean b.p. 50 mm Hg. Approximately 130 mm saline infused.

Sample No.		1	2	3	4
Experimental conditions		Control	20 µg/kg adrenaline i.v.	Control	25 µg adrenaline into caudate nucleus
time (min.)		0	11	57	73
plasma (g)		5.7	8.2	6.7	7.1
collection time (min.)		4	4	4	4
Cortisol	µg/g adr./min.	11.5	6.4	8.4	9.6
	µg/hr/adr./kg b. wt.	34.4	19.2	25.5	28.6
Corticosterone	µg/g adr./min.	7	6.7	6.7	7
	µg/hr/adr./kg b. wt.	20.9	19.9	19.9	20.9
Ratio F/B		1.65	0.96	1.25	1.37
NaOH-fluorescence		x:+ A:++	x:? A:++	x:? A:+	x:? A:+++

TABLE 13

Cat 2 - female - 2.9 kg. Influence of adrenaline on the cortisol and corticosterone secretion of the denervated left adrenal (218 mg). Denervation 12 days prior to the experiment. Mean b.p. 80 mm. Hg. Approximately 40 ml. saline infused.

Sample No:		1	2	3	4
Experimental conditions		Control	28 µg/kg Adrenaline intravenously	Control	25 µg Adrenaline into lat. ventr.
time (min.)		0	18	66	81
plasma (g)		2.7	4.8	1.9	2.3
Collection time (min.)		8	8	8	8
Cortisol	µg/g adr./min.	12.0	12.0	8.3	10.9
	µg/hr/adr./kg b. wt.	52.0	52.0	35.8	47.2
Corticosterone	µg/g adr./min.	8.4	7.3	4.6	4.3
	µg/hr/adr./kg b. wt.	36.5	31.5	19.9	18.6
Ratio F/B		1.43	1.65	1.8	2.54
NaOH-fluorescence		x:+ A:+	x:- A:-	x:+ A:++	x:++ A:++

TABLE 14

Cat 3 - male - 4.8 kg. Influence of adrenaline on the cortisol and corticosterone secretion of the denervated left adrenal (250 mg). Denervation 16 days prior to the experiment. Mean b.p. 60 mm Hg. Approximately 50 ml. saline infused.

Sample No.:		1	2	3	4
Experimental conditions		Control	31 µg/kg Adrenaline i.v.	Control	25 µg adrenaline into brain tissue near left lat. ventr.
time (min.)		0	25	69	84
plasma (g)		1.1	1.8	1.5	2.0
collection time (min.)		4	4	4	4
Cortisol	µg/g adr./min.	4.5	3.1	5	5.5
	µg/hr/adr./kg b. wt.	13.9	9.6	15.3	17.0
Corticosterone	µg/g adr./min.	4.9	0.8	5	5.8
	µg/hr/adr./kg b. wt.	15.2	2.5	15.3	17.9
Ratio F/B		0.92	3.9	1.0	0.95
NaOH-fluorescence		X:- A:+	X:- A:-	X:- A:+	X:- A:++

TABLE 15

Cat 4 - male - 5.1 kg. Effect of an adrenaline infusion on cortisol and corticosterone secretion of the denervated right adrenal (365 mg). Denervation 10 days prior to the experiment. Mean b.p. 40 mm Hg. Approximately 100 ml. saline infused.

Sample No.		1	2
Experimental conditions		Control	87 µg/kg adrenaline i.v.
time (min.)		0	45
plasma (g)		8.2	8.3
collection time (min.)		4	4
cortisol	µg/g adr./min.	8.5	7.4
	µg/hr/adr./kg b. wt.	36.2	31.5
corticosterone	µg/g adr./min.	6.7	5.8
	µg/hr/adr./kg b. wt.	28.6	24.5
Ratio F/B		1.27	1.28
NaOH-fluorescence		x:? A:+	x:? A:+

weight of the right adrenal was in some instances, greater in others smaller than that of the left adrenal. The figures per kg body weight were especially low in the last two animals which had no right adrenal.

Infusion of adrenaline remained without significant influence on the secretion rate of corticosterone.

The histological picture showed in all instances, packets of typical adrenocrotical tissue, the cells more or less rich in lipids. None of the regenerates contained detectable amounts of chromaffin tissue.

2. Larger animals

a. Cats. From the amount of cortisol and corticosterone present in the control samples a mean secretion rate per g adrenal/min. of 8.3 ± 1 µg cortisol and 6.2 ± 0.5 µg corticosterone can be calculated for the denervated cat adrenal. Expressed in µg/adrenal/hr/kg b. wt. the secretion rate was found to be 30.4 ± 5.3 for cortisol and 23.8 ± 1.9 for corticosterone. For both glands and per day the secretion rate amounted to 1.46 ± 0.25 mg of cortisol and 1.13 ± 0.09 mg of corticosterone. Bush (1953) estimated with the semiquant-

TABLE 16

Dog - male - 12.2 kg. Influence of adrenaline on the cortisol and corticosterone secretion of the denervated left adrenal (535 mg). Mean b.p. 100 mm Hg.

Sample No:		1	2	3	4	5
Experimental conditions		Control	11.7 µg/kg adrenaline i.v.	Control	50 µg adrenaline into lat. ventr.	Control
time (min.)		0	9	48	65	89
plasma (g)		2.7	4.5	4.4	3.4	1.5
collection time		5	5	5	5	5
Cortisol	µg/g adr./min.	9.7	11.2	6.2	7.5	5.5
	µg/hr/adr./kg b. wt.	25.2	28.1	15.6	18.9	13.75
Corticosterone	µg/g adr./min.	2.7	4.8	3.9	4.7	-
	µg/hr/adr./kg b. wt.	6.9	12.8	9.7	11.8	-
Ratio F/B		3.6	2.3	1.6	1.6	-
NaOH-fluorescence		x:?	x:?	x:-	x:?	x:-

itative NaOH-fluorescence method (see p. 25) a secretion rate of 3.5 - 8.5 mg Δ^4-3-ketosteroids/kg b. wt./24 hr in cats with innervated adrenals. Considering that Bush's figures include the notable amounts of 11-dehydrocorticosterone and 17-ketosteroids which cat adrenals produce, the secretion rate of a denervated cat adrenal cortex does not seem to be very different from that of an innervated one.

Contrary to expectation, the intravenous infusion of adrenaline did not cause an increase in the amounts of cortisol and corticosterone secreted. There was rather a tendency towards decrease of the secretion rate. After intraventricular or intracerebral adrenaline injections a slightly increased glucocorticoid secretion was noticable. The mean increase was about 15%.

b. Dog. During the control periods, the acutely denervated dog adrenal secreted 7.1 ± 1.4 µg cortisol and 3.3 ± 0.7 µg corticosterone per g tissue. The secretion amounted to 18.2 ± 3.9 µg cortisol and 8.3 ± 1.8 µg corticosterone per adrenal per hr per kg b. wt. The amount secreted per hour per adrenal lies within the range of the figures obtained with a similar method by Hechter et al. (1955) in dogs whose adrenals were not de-

TABLE 17

Monkey 1 - male - 5 kg. Influence of adrenaline on the cortisol and corticosterone secretion of the denervated left adrenal (350 mg). Denervation 6 days prior to the experiment. Mean b.p. 30 mm. Hg. Approximately 75 ml. saline infused.

Sample No:		1	2	3	4	5	6	7
Experimental conditions		Control	Control	8.5 µg/kg adrenaline i.v.	Control	2.1 µg/kg adrenaline i. carot.	Control	5 µg adrenaline into lat.vent
time (min.)		0	5	17	56	63	104	123
plasma (g)		7.5	6.1	8	6.5	5.2	5.2	3.3
collection time (min.)		4	4	4	4	4.2	4	4
cortisol	µg/g adr./min.	13.3	13.3	15.7	13.6	11.5	14.2	15.1
	µg/hr/adr./kg b. wt.	55.8	55.8	66.0	57.0	43.3	59.8	63.5
corticosterone :	µg/g adr./min.	2.7	-	1.4	2.5	1.7	-	2.1
Ratio F/B		4.9	-	11.2	5.4	6.8	-	7.2
NaOH-fluorescence		X:- E:+	X:+ E:+	X:- E:+	X:+ E:+	X:+ E:?	X:++ E:+	X:+ E:?

TABLE 18

Monkey 2 - male - 5.1 kg. Influence of adrenaline on cortisol secretion of the denervated left adrenal (320 mg). Denervation 13 days prior to the experiment.

Sample No:		1	2	3	4	5	6
Experimental conditions		Control	Control	6 µg/kg adrenaline i.v.	Control	58 µg adrenaline into lat. ventr.	Control
time (min.)		0	9	16	79	106	118
plasma (g)		6.1	2.5	5.4	5.5	4.5	5
collection time (min.)		3	1.5	2.25	3	2.25	2.5
Corti-sol	µg/g adr./min.	19.8	19.8	23.8	19.8	17.7	22.5
	µg/hr/adr./kg b. wt.	74.5	74.5	89.5	74.5	66.7	84.8
NaOH-fluorescence		x:+ B:+	x:? B:+	x:- B:+	x:- B:+	x:- B:+	x:- B:+

nervated.

Intravenous infusion of adrenaline caused a 15% increase of the cortisol and a 77% increase of the (very low) corticosterone secretion. After injection of 50 µg adrenaline into the lateral ventricle of the brain a 20% increase of the cortisol and corticosterone secretion were observed. It is doubtful whether these changes are significant.

c. Monkeys. The mean secretion rate of denervated monkey adrenals was calculated as 17.0 ± 1.1 µg cortisol/g adrenal/min. and 67.2 ± 4.8 µg cortisol/adrenal/hr/kg b. wt. The amount of corticosterone secreted in 1.5 - 4.2 min. was at the threshold of the method of estimation. The figure for secretion rate is also comparable with the figures obtained by Bush (1953) in normal monkeys.

After intravenous infusion of adrenaline an increase of the cortisol secretion of about 20% occurred in both monkeys. In monkey 1 the cortisol secretion was decreased by 15.4% after intracarotid infusion of adrenaline and increased by 6% after 5 µg adrenaline had been injected into the lateral ventricle of the brain. In monkey 2 intra-

ventricular injection of 5 µg adrenaline caused a decrease in the output of cortisol by 10.5%. The significance of the observed changes in the secretion rates is very doubtful, particularly since the blood pressure was extremely low.

was followed by

Discussion

The experiments reported have shown that under conditions of operative stress in the rat, cat, and monkey, the secretion rate of corticosterone and cortisol per g adrenal tissue is not significantly changed (1) by demedullation or denervation of the adrenal gland and (2) by infusing or injecting adrenaline.

From finding (1) it can be concluded that, in the species investigated, the presence of an innervated adrenal medulla is not essential for the secretion of corticosterone and cortisol under conditions of operative stress. Thus medullary amines do not appear to play an indispensable role in the mechanism which leads to ACTH release in the course of operative stress.

The second observation is less easy to interpret. An influence of exogenous adrenaline on adrenocortical secretion by either direct stimulation of the adrenal cortex or by release of ACTH appears to be well established by the data reported in the literature. Thus, infusion of physiological quantities of adrenaline in dogs and cats (Vogt, 1944) elicited a significant increase in adrenocortical secretion, as tested by the Selye-

Schenker test. Since a significant difference is rarely seen in the Selye-Schenker test unless one sample contains about three times as much hormone as the other (Vogt, 1943) the increase in hormonal output caused by adrenaline represented a rise of several hundred per cent. In the present experiments, no such increase was observed when the cortisol and corticosterone output was measured by chemical means.

One possible explanation for this discrepancy is the presence in adrenal vein blood of very small amounts of a corticosteroid which is highly active in the Selye-Schenker test, but escaped detection by the chemical method. Part of the difference might also be accounted for by variation in release of ACTH due to the operation as such.

Under the conditions of our experiments, corticosterone and cortisol secretion may already have been maximal before the infusion owing to release of large amounts of endogenous ACTH (compare Chapter 4). If this was the case, one could not expect to observe a stimulating influence of adrenaline on cortical secretion rate which is mediated by the anterior pituitary. This suspicion is supported by the fact that the cats and monkeys were in a shocked state. Apparently, cannulation

of the adrenal vein in an animal deprived of its splanchnic nerves several days previously is less well tolerated than the same operation performed immediately after an acute splanchnectomy as practised in Vogt's earlier experiments, and in which, therefore, corticoid secretion was still susceptible to stimulation.

Summary

Corticosterone secretion was measured in the rat after adrenal demedullation; cortisol and corticosterone secretion were estimated in the denervated adrenal of the cat, the dog and the monkey. In these animals, infusion of adrenaline had no significant effect on the secretion rate of these steroids. The results in the rat, cat and monkey might be interpreted by assuming that secretion of ACTH was maximal before the infusion was begun. The single experiment in the dog in which a small increase in secretion was seen, requires confirmation on a larger material.

CHAPTER 7

Inhibitory Action of Oestrogens on the Corticosterone Secretion of Rat Adrenals in vivo

Many investigators have observed that female sex hormones and some of their synthetic substitutes, produce adrenal hypertrophy in adult male rats (Korenchevsky and Dennison, 1935; Selye, Collip and Thomsen, 1935; Noble, 1939, etc.). This hypertrophy is accompanied by a diminution of the lipid content as was first seen by Hall (1938), following treatment with oestradiol, and by Loeser (1939) following stilboestrol. The lipid loss is especially pronounced after administration of small doses of hexoestrol (Vogt, 1945).

These changes in the adrenal gland occur only when the pituitary gland is intact. Adrenals of hypophysectomized rats maintained on ACTH do not undergo specific morphological changes after oestrogen administration (Selye and Collip, 1936). During hexoestrol treatment the pituitary grows for at least 6 weeks (Somers, 1948) and the amount of circulating ACTH in rats injected with oestradiol was found to be increased (Gemzell, 1952).

The functional state of an adrenal deprived of its lipids by oestrogens was a matter of conjecture, until Vogt (1955) measured the amount of cortico-

sterone secreted into adrenal vein blood of hexoestrol treated rats after laparotomy in urethane anaesthesia. She found it greatly diminished. The inhibition was already significant after two daily injections. The effect of hexoestrol on adrenocortical secretion is completely reversible. As an explanation for the mode of action it was postulated that hexoestrol interferes with some step of cholesterol synthesis, assuming that cholesterol is the main precursor of corticosterone. The theory obtained strong support from the observation of Boyd and McGuire (1955), that the in vitro cholesterol synthesis from C^{14}-acetate by liver slices was considerably reduced when the liver was taken from rats pretreated with hexoestrol.

The hypertrophy of the adrenals was suggested to be analogous to the goitre caused by antithyroid substances. As a consequence of the decreased corticosterone secretion the amounts of circulating ACTH are increased and stimulate the growth of the adrenal cortex.

The present work deals with the question whether the observed inhibition of adrenocortical secretion is peculiar to hexoestrol or whether it is also produced by other synthetic substances with oestrogenic properties or by naturally occurring oestrogens, which elicit similar structural changes

in the adrenals. The substances studied were chlorotrianisene, ethinyloestradiol and oestradiol-17β. An additional series of experiments was made with hexoestrol. Chlorotrianisene was selected because its structure bears no relation to the stilbene derivatives.

Methods

Hexoestrol (B.D.H.): 32 rats of different origin were injected subcutaneously for four to six days with 0.4 mg hexoestrol/kg/day (0.2% solution in arachis oil), (see Table 19).

Ethinyloestradiol: 4 rats (O-strain) received five daily subcutaneous injections of 2 mg ethinyloestradiol/kg (1% solution in propylene glycol), 2 control rats of the same strain 0.2 ml propylene glycol/kg/day for five days (see Table 20).

Chlorotrianisene (TACE*, tri-p-anisylchloroethylene): The dosage used in all experiments was 40 mg/kg/day. For experiments, in which the drug was fed, a 1% colloidal solution was prepared in 50% propylene glycol. 1 ml of propylene glycol was added to every 20 mg of TACE, the mixture heated in a boiling water bath until TACE was dissolved and equal amount of water added under constant stirring to the hot solution. The milky fluid was fed by stomach tube within the next 20 minutes.

* Trade mark of The Wm. S. Merrell Company

For subcutaneous injections a 5% solution in arachis oil was prepared.

Experiments were carried out on 8 groups of rats. Five groups were treated in different ways with TACE, 3 served as controls and were treated in a corresponding fashion with the vehicle only.

Group 1: 4 rats (T-,B-, and O-strain); fed for five days with TACE.

Group 2: 4 rats (T-,B-, and O-strain); fed for five days with 50% propylene glycol (4 ml/kg), (Table 21).

Group 3: 11 rats (T-,E-, and O-strain); fed for 15 days with TACE.

Group 4: 4 rats (T-strain) fed for 15 days with 50% propylene glycol (4 ml/kg), (Table 22).

Group 5: 10 rats (B-strain); 15-17 daily injections of TACE in arachis oil (Table 23).

Group 6: 14 rats (O-,B-, and E-strain); 13-15 daily injections of 0.8 ml arachis oil/kg (Table 24).

Group 7: 3 rats (T-strain); fed with TACE and simultaneously injected with arachis oil for 5 days (Table 21).

Group 8: 5 rats (B-strain); treated in the same fashion as the rats of Group 7, but for 15 days (Table 23).

Oestradiol-17β (henceforth called oestradiol)

Experiments were carried out on 5 groups of rats. The groups were treated as follows:

Group 1: 6 rats (T-strain); 5 daily subcutaneous injections of 0.8 mg oestradiol/kg (0.33% solution in arachis oil).

Group 2: 4 rats (T-strain); 2 mg oestradiol/kg/day for ten days, during the first four days as one injection per day (0.2 ml/kg of a 1% solution in propylene glycol), during the last six days as two injections per day (0.2 ml/kg of a 0.5% solution in propylene glycol).

Group 3: 4 rats (E-, O-, and T-strain); 5 daily injections of 2 mg oestradiol/kg (1% solution in propylene glycol (Table 25).

Group 4: 5 rats (O-strain); 5 daily injections of 0.2 ml propylene glycol/kg.

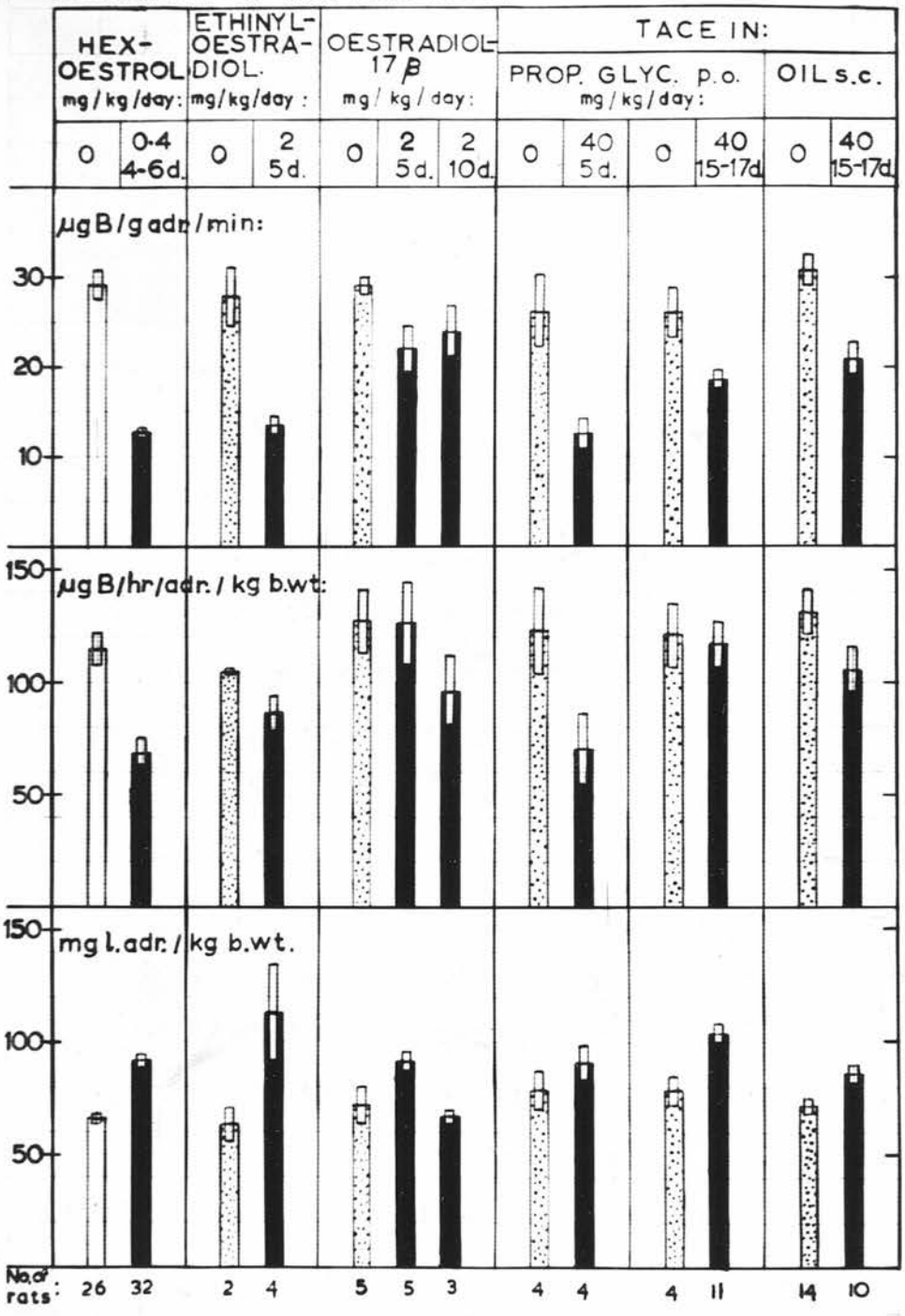
Group 5: 5 rats (O-strain); 5 daily injections of 2 mg oestradiol/kg (1% solution in propylene glycol) (Table 26).

In all experiments, adrenal vein blood was collected in pentobarbitone anaesthesia 16-24 hours after the last drug administration. The plasma was subjected to the extraction and chemical estimation procedure outlined in the chapter on general methods.

Figure XII

FIG.

INHIBITION BY OESTROGENS OF THE CORTICOSTERONE SECRETION OF RAT ADRENALS.



□ : UNTREATED CONTROL RATS.

▨ : CONTROL RATS, TREATED WITH VEHICLE.

■ : OESTROGEN TREATED RATS.

Results

The observed effects of different oestrogens on the secretory capacity of rat adrenals are summarised in Figure XII.

Hexoestrol

Data obtained on the function of rat adrenals after pretreatment of the animals with hexoestrol, are listed in Table 19. Whereas the mean corticosterone secretion of 26 normal rats was estimated as 29.1 µg/g adrenal/minute, it was only 12.1 µg/g adrenal/minute in the 36 rats pretreated with 4-6 daily injections of hexoestrol. The hypertrophy of the adrenals was not sufficient to provide normal corticosterone secretion per kg body weight. In only 2 out of 32 hexoestrol treated animals did a large fluorescent spot appear in the x-region of the chromatograms. Microscopic inspection of the sudan-stained glands invariably showed lipid depletion in the zona fasciculata. The degree of lipid depletion could be correlated with the degree of functional inhibition in most of the adrenals. Usually no lipid depletion in the zona glomerulosa appeared after this short treatment. All animals lost weight (between 8 and 73 g), but there was no correlation between the amount of weight lost and the degree of adrenal inhibition. The lower dia-

TABLE 19

"Hexoestrol"

Cortical secretion of the left adrenal of rats pretreated with 4-6 daily injections of hexoestrol (0.4 mg/kg b.wt. s.c., 0.2% solution in arachis oil). Blood collected in pentobarbitone anaesthesia.

Date	Rat No.	Strain	No. of injections	b.wt. (g) at start	b.wt. (g) at end	Adrenal plasma (g)	mean body temp. (°C)	l. adrenal mg/kg b.wt.	Corticosterone secretion µg/g adr./min.	Corticosterone secretion µg/hr/adr./kg b.wt.	% recovery	NaOH fluorescence
(n=26)	Controls in pentobarbitone Groups 2 + 3 (see Tables 4 & 5)						mean ± S.E.	66±1.9	29.1±1.6	114.9±6.9		
22.6 1955	106	B	5	390	357	2.2	37	79	14.1	67.0	72	x:+
27.6 1955	107	B	5	325	293	2	35.5	114	10.8	66.9	75	x:+
30.6 1955	108	B	5	320	262	0.9	36.5	103	9.0	61.0	72	x:+
30.6 1955	109	B	5	362	336	1.3	39	89	13.4	72.0	72	x:+
4.7 1955	110	B	4	367	305	2.5	37.5	106	10.0	64.3	85	x:run off
4.7 1955	111	B	4	358	301	1.5	38	117	11.9	84.0	85	x:run off
4.7 1955	112	B	5	315	272	1.3	38	118	7.3	51.4	62	-
10.1 1956	138	B	5	428	400	2	-	75	13.2	59.3	61	x:-
12.1 1956	142	B	5	406	357	2	38	75	12.8	57.2	61	x:-
12.1 1956	143	B	5	358	332	2.3	-	91	9.3	50.7	76	x:+
23.1 1956	145	B	5	403	353	2.5	36	83	19.2	95.2	72	x:+
26.1 1956	148	B	6	320	253	1.2	?	135	11.3	91.7	60	x:+
26.1 1956	149	B	6	325	252	3.3	34.5	131	9.9	77.8	69	x:+

CONTD:

TABLE 19 Contd:

Date	Rat No.	Strain	No.of injections	b.wt. (g) at start	b.wt. (g) at end	Adrenal plasma (g)	mean body temp. (°C)	1: adrenal mg/kg b.wt.	Corticosterone secretion µg/g adr./min	Corticosterone secretion µg/hr/ adr./kg b.wt.	% recovery	NaOH fluorescence
26.1 1956	151	B	6	365	300	2	35	93	9.8	59.7	62	x:-
6.2 1956	159	B	5	355	320	-	40	72	11.8	51.3	65	x:-
9.2 1956	160	B	5	310	247	0.8	39	96	8.2	47.0	63	x:-
9.2 1956	161	B	5	400	340	2.8	32.5	88	11.6	61.3	-	x:-
9.2 1956	162	B	5	358	313	1.7	34.5	86	17.6	90.8	72	x:+
9.2 1956	163	B	5	430	365	2.5	35	99	14.1	83.5	69	x:-
9.2 1956	164	B	5	401	344	1.2	37	79	20.9	98.8	67	x:+
3.5 1956	193	T	4	310	270	1.4	39	92	19.6	108	82	x:+
3.5 1956	194	T	4	298	264	1.5	33.5	74	18.0	80.4	70	x:?
14.5 1956	207	T	4	-	255	2.6	36	106	19.8	126.0	65	x:++
14.5 1956	208	T	4	-	251	1.7	35	78	11.9	55.8	64	x:?
21.5 1956	218	B	5	373	338	2.7	39.5	89	6.2	33.1	64	x:++
21.5 1956	219	B	5	333	306	2.4	36.5	86	8.4	43.1	72	x:+
21.5 1956	220	B	5	358	350	2.1	38.5	101	11.5	69.8	70	x:+
11.6 1956	239	B	5	430	408	2.1	34.5	47	15.1	42.1	100	x:+

CONTD:

TABLE 19 Contd:

Date	Rat No.	Strain	No. of injections	b.wt. (g) at start	b.wt. (g) at end	Adrenal plasma (g)	mean body temp. (°C)	L. adrenal mg/kg b.wt.	Corticosterone secretion µg/g adr./min	Corticosterone secretion µg/hr/ adr./kg b.wt.	% recovery	NaOH fluorescence
11.6 1956	240	B	5	432	372	2.3	36.5	93	11.8	66.0	71	x:-
11.6 1956	241	B	5	430	406	2.4	39.5	80	9.5	45.3	79	x:?
2.7 1956	269	0	4	418	396	2.9	-	61	15.6	57.6	77	x:?
2.7 1956	270	0	4	363	318	1.7	36.2	92	13.8	76.8	69	x:+
			mean ± S.E.:					91.5±3.0	12.7±0.46	68.6±5.96		
Significance of difference from controls:								$P < 0.01$	$P < 0.01$	$P < 0.01$		

TABLE 20

Ethinyloestradiol

Cortical secretion from the left adrenal of rats, given 5 injections of ethinyloestradiol in propylene glycol and of 5 controls injected with

Blood coll. Date	Rat No.	Treatment	b.wt. (g) at start	b.wt. (g) at end	adrenal plasma (g)	Mean body temp. (°C)	l.adrenal mg/kg b. wt.
24.5 1956	221	Controls: 0.2 ml propylene glycol/ kg b. wt./day s.c. for 5 days	317	334	1.8	36	57.2
24.5 1956	222		328	335	2.5	37.5	69.6
		Mean ± S.E.:					63.7 ± 7.7
24.5 1956	223	Ethinyloestradiol 2 mg/kg b.wt./day s.c. (1% solution in propylene glycol) for 5 days	312	270	2.2	38	178.5
24.5 1956	224		325	292	3.1	34	87.0
24.5 1956	225		347	294	3.2	36	90.8
24.5 1956	226		330	296	2.3	37	96.4
		mean ± S.E.					113.2 ± 22.2
Significance of difference from controls:							[P > 0.1]

TABLE 20 Contd:

propylene glycol only. (O-strain, blood collected over a 15 min. period in pentobarbitone anaesthesia.)

Rat No.	Corticosterone secretion		% re-covery	NaOH fluor-escence	Histology
	µg/g adr./min	µg/hr/adr./kg b.wt.			
221	30.4	104.03	69	x:+	normal
222	25.2	105.00	76	x:+	normal
	27.8± 3.26	104.5±0.6			
223	10.9	109.0	69	x:-	severe lipid depletion in fasciculata and glomerulosa
224	14.7	76.8	76	x:-	lipid depletion in fasciulata
225	14.2	77.6	76	x:?	severe lipid depletion in fasciculata
226	14.05	81.0	-	x:+	lipid depletion in fasciculata
	13.5±0.95	86.1±7.75			
	$P < 0.01$	$P < 0.1, > 0.05$			

grams in Figure IX do not indicate a correlation between secretory capacity and body weight. The hypertrophy of the adrenals seems however, to be more pronounced in smaller animals.

Ethinyloestradiol

The results of experiments on the effect of ethinyloestradiol on the secretory capacity of rat adrenals are presented in Table 20. They show that ethinyloestradiol caused an inhibition of adrenocortical function accompanied by a lipid loss in the glands. The mean amount of corticosterone secreted by the left adrenal was 13.5 µg/g adrenal/minute. When expressed in µg/hr/adrenal/kg body weight the corticosterone secretion was depressed by about 25% in 3 out of 4 animals. In these rats, the left adrenals were about 45% heavier than those of the controls. In the fourth rat (No.223), the adrenal was excessively enlarged and, although only 10.9 µg corticosterone were secreted per g adrenal/minute, the secretion per kg body weight lay within the normal range. A clear spot in the x-region was only visible in the chromatogram of the plasma extract of rat No.226. The microscopic inspection of the sudan-stained adrenals showed lipid depletion in the zona fasciculata. With the exception of No.223, the glomerulosa was not lipid depleted. In all

TABLE 21

"TACE" short treatment

Cortical secretion of the left adrenal of rats, pretreated with 5 daily oral doses of chlorotrianisene (TACE) in propylene glycol (Group b) and of control rats given the vehicle only

Group	Blood coll. Date	Rat No.	Strain	Treatment	b.wt.(g) at start	b.wt.(g) at end	adrenal plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b.wt.
a	17.5 1956	209	T	4 ml 50% propylene glycol/kg b.wt. fed for five days.	274	275	1.9	36.5	74
	21.5 1956	215	B		329	332	1.6	34	67
	14.6 1956	242	0		407	387	1.8	36	72
	14.6 1956	243	0		304	295	1.1	37.5	103
				mean ± S.E.:					79 ± 8.8
b	17.5 1956	210	T	40 mg TACE/kg b.wt. (1% sol. in 50% propylene glycol) fed for five days.	288	259	2.1	38.5	113
	21.5 1956	216	B		359	317	-	35.5	78
	21.5 1956	217	B		361	345	2.2	35.5	85
	14.6 1956	246	0		402	375	-	36	89
				mean ± S.E.:					91 ± 8.5
	Significance of difference from controls:								P > 0.1
c	19.7 1956	275	T	TACE as in group b, and, in addition, daily 0.8 ml arachis oil/kg b.wt. s.c.	290	268	1.5	34.5	90
	19.7 1956	276	T		282	271	2	33.5	88
	19.7 1956	277	T		281	270	2	35.5	83
				mean ± S.E.:					87 ± 2.4
	Significance of difference from TACE, group b								P > 0.1

TABLE 21 Contd:

(Group a). The rats in Group c received in addition to TACE 5 daily s.c. injections of arachis oil. Blood collected in pentobarbitone anaesthesia over a 15 min. period.

Rat No.	Corticosterone secretion		% re-covery	NaOH fluor-escence	Histology
	µg/g adr./min	µg/hr/adr./kg b. wt.			
209	34.0	150	75	x:-	normal
215	17.7	71	71	x:-	some lipid depleted patches in fasciculata
242	30.0	129	67	x:+	normal
243	23.0	142	69	x:+	normal
	26.2 ± 4	123 ± 19			
210	17.1	116	69	x:-	(outer fasciculata lipid depletion in
216	12.4	58	74	x:+	lipid depletion in glomerulosa and fasciculata
217	9.7	50	67	x:+	lipid depletion in glomerulosa and fasciculata
246	10.7	57	69	x:++	severe lipid depletion in fasciculata
	12.5 ± 1.8	70 ± 16			
	P=0.02-0.01	P < 0.1			
275	14.3	78	66	x:++ A:?	some lipid depletion in fasciculata
276	14.8	78	76	x:++ A:?	some lipid depletion in fasciculata
277	16.0	80	71	x:+	severe lipid depletion in fasciculata
	15 ± 0.6	78.7±0.7			
	P > 0.1	P > 0.1			

glands large venous sinuses were visible in the inner fasciculata and in the reticularis, but there were no haemorrhages or necroses. Five daily injections of 0.2 ml propylene glycol/kg body weight did not influence the microscopic anatomy or the lipid content of the glands. The control rats also gained in body weight during the injection period. The ethinyloestradiol treated rats lost between 33 and 53 g of their initial body weight during the period of injections.

Chlorotrianisene

The results obtained from rats pretreated with TACE are presented in Tables 21, 22, 23 and 24.

In all experiments, TACE elicited inhibition of secretory capacity, lipid loss and hypertrophy of the adrenals. The mean corticosterone secretion of the left adrenals of rats fed for 5 days with TACE was 12.5 µg/g adrenal/minute, that of the control group fed with propylene glycol 26.2 µg (see Table 21). The secretion rate per kg body weight was also diminished, but because of large individual variations, this difference was not significant. The mean adrenal weights of the rats of the control group was somewhat higher than that of normal rats. There was a marked lipid loss in

TABLE 22

"TACE" long treatment

Cortical secretion of the left adrenal of rats pretreated with 15 or 17 daily oral doses of chlorotrianisene (TACE) in propylene

Date	Rat No.	Strain	Treatment	Days of treatment	b.wt. (g) at start	b.wt. (g) at end	adrenal plasma (g)	mean body temp. (°C)	l-adrenal mg/kg b.wt.
7.5 1956	195	T?	Controls: 4 ml 50% propylene glycol/kg b. wt. fed daily	17	333	337	1.3	34	74
7.5 1956	196	T?		17	320	332	1.2	35	72
28.5 1956	227	T		15	265	273	1.1	33.5	97
28.5 1956	228	T		15	323	319	1.2	38	71
			mean±S.E.:						78.5±6.3
7.5 1956	197	T?	TACE: 40 mg TACE/kg b. wt. fed daily (as 1% soln in 50% propylene glycol)	17	341	293	1.3	34	86
7.5 1956	198	T?		17	320	273	1.3	?	116
28.5 1956	229	T		15	271	230	2.4	33.5	125
28.5 1956	230	T		15	285	254	2.3	40	109
28.6 1956	262	E		15	325	284	1.9	37	97
28.6 1956	263	E		15	335	298	1.5	?	94
28.6 1956	264	E		15	306	260	1.4	37	107
4.10 1956	312	B		15	280	234	1.5	37.5	118
4.10 1956	313	B		15	298	262	2.3	36.5	91
11.10 1956	324	B		15	280	208	1.3	36.5	102
11.10 1956	325	B		15	294	256	1.4	39	99
			mean±S.E.:						104±3.7
Significance of difference from controls:									$P < 0.01$

TABLE 22 Contd:

glycol, and of control rats pretreated with the vehicle alone. Blood collected in pentobarbitone anaesthesia for 15 min.

Rat No.	Corticosterone secretion µg/g adr./min	Corticosterone secretion µg/hr/adr./Kg b.wt.	% recovery	NaOH fluorescence	Histology
195	22.0	97	74	x:++	lipid depletion in glomerulosa and fasciculata
196	21.9	94	82	x:++	lipid depletion in glomerulosa:some in fasciculata
227	26.2	152	81	x:?	some lipid depletion in glomerulosa
228	33.3	141	76	x:?	normal
	25.9 ± 2.8	121 ± 14.1			
197	15.9	82	85	x:+	some lipid depletion in glomerulosa and fasciculata
198	14.7	103	88	x:+	some lipid depletion in glomerulosa
229	24.6	185	87	x:+	lipid depletion in glomerulosa:some in fasciculata
230	16.7	125	78	x:+	severe lipid depletion in glom.: " " " "in fasciculata
262	19.9	116	78	x:+ A:?	severe lipid depletion in glomerulosa
263	16.2	92	69	x:+	do. do. and outer fasciculata
264	17.2	110	65	x:+ A:?	do. do. some also in fasciculata
312	19.0	135	70	x:?	lipid depletion in fasciculata
313	20.1	110	65	x:?	lipid depletion in fasciculata
324	22.7	139	71	x:+	some lipid depletion in fasciculata
325	16.1	95	73	x:+	lipid depletion in fasciculata
	18.46±0.94	117 ± 9.8			
	$P < 0.01$	$P > 0.1$			

TABLE 23

TACE and oil combined

Cortical secretion of the left adrenal of rats (B-strain) pretreated either with 15-17 daily s.c. injections of chlorotrianisene

Blood coll. Date	Rat No.	Treatment	Days of treatment	b.wt. (g) at start	b.wt. (g) at end	adrenal plasma (g)	mean body temp. (°C)	l.adrenal mg/kg b.wt.
(n=11)		TACE-controls: 15-17daily oral doses in propylene glycol, see Table 22		mean ± S.E.:				104 ± 3.7
(n=14)		Oil-controls:13-15 daily s.c. injections of arach. oil, see Table 24		mean ± S.E.:				71.7± 3.5
20.2 1956	169	40 mg TACE/ kg. b. wt./ day s.c. (5% solution in hot ol. arach.)	15	373	315	1.5	33	74
20.2 1956	170		15	431	356	1.1	35	84
20.2 1956	171		15	326	266	1.5	34	85
20.2 1956	172		15	338	278	1.9	36.5	74
27.2 1956	176		17	285	276	2.1	33.5	83
27.2 1956	177		17	303	292	1.5	35.5	80
27.2 1956	178		17	300	220	2	36	95
27.2 1956	179		17	313	265	2	36.5	78
15.3 1956	180		15	317	274	1.1	35.5	112
15.3 1956	181		15	358	283	1.7	35.5	88
		mean ± S.E.:						86 ± 3.8
Significance of difference from TACE-controls:								P < 0.01
Significance of difference from Oil-controls:								P < 0.05

CONTD:

TABLE 23 Contd:

(TACE) in arachis oil or with 15 days oral chlorotrianisene in propylene glycol combined with daily s.c. injections of arachis oil. Blood collected in pentobarbitone anaesthesia over a 15 min. period.

Rat No.	Corticosterone secretion µg/g adr./min.	Corticosterone secretion µg/hr/adr. kg b. wt.	% recovery	NaOH-fluorescence	Histology
	18.46 ± 0.94	117 ± 9.8			
	30.7 ± 1.7	131 ± 10			
169	11.8	52	70	x:-	normal
170	11.0	56	79	x:?	lipid depleted patches in fasciculata
171	21.3	108	73	x:?	normal
172	25.7	114	75	x:+	severe lipid depletion in fasciculata
176	21.9	109	69	x:+	some lipid depletion in fasciculata
177	23.4	112	65	x:+	some lipid depletion in fasciculata
178	26.2	149	59	x:-	some lipid depletion in fasciculata
179	28.8	136	60	x:+	some lipid depletion in fasciculata
180	20.1	135	71	x:+	severe lipid depletion in fasciculata
181	17.4	92	68	x:+	severe lipid depletion in fasciculata
	20.8 ± 1.8	106.2±10			
	$P > 0.1$	$P > 0.1$			
	$P < 0.01$	$P < 0.1$			

CONTD:

TABLE 23 Contd:

Blood coll. Date	Rat No.	Treatment	b.wt. (g) at start	b.wt. (g) at end	adrenal plasma (g)	mean body temp. (°C)	l.adrenal mg/kg b. wt.
4.10 1956	308	40 mg TACE daily kg. b. wt. by mouth (1% solution in 50% propylene glycol) + 0.8 ml ol. arach./kg b. wt. s.c. for 15 days.	300	286	1.4	36	129
4.10 1956	309		292	257	1.5	37	130
8.10 1956	314		279	255	1.6	37	99
8.10 1956	315		305	265	1.6	37.5	85
11.10 1956	320		290	272	1.8	35.5	91
11.10 1956	321		290	287	2.2	38.5	89
mean ± S.E.:							104 ± 7.3

Significance of difference from TACE controls (Group 2, Table 22) $P > 0.1$

TABLE 23 Contd:

Rat No.	Corticosterone secretion µg/g adr./min	Corticosterone secretion µg/hr/adr./kg b. wt.	% recovery	NaOH fluorescence	Histology
308	10.1	79	65	x:?	lipid depletion in glomerulosa and fasciculata
309	13.2	103	69	x:?	lipid depletion in glomerulosa and fasciculata
314	17.5	108	70	x:+	severe lipid depletion in glomerulosa: lipid depletion in fasciculata
315	20.1	103	71	x:?	severe lipid depletion in glomerulosa and fasciculata
320	16.1	88	66	x:+	lipid depletion in glomerulosa and fasciculata
321	22.7	121	70	x:+	some lipid depletion in fasciculata
	16.6 ± 2	99.5 ± 6.8			
	$P > 0.1$	$P > 0.1$			

the zona fasciculata and twice in the zona glomerulosa of the TACE treated rats. The loss in body weight during the period of treatment ranged from 16 to 42 g.

The results of more prolonged treatment are found in Table 22.

The mean corticosterone secretion of the left adrenal of rats fed for 15-17 days with TACE was 18.5 µg/g adrenal/minute, that of the control rats, fed for the same length of time with propylene glycol 25.9 µg. The difference is significant. Corticosterone secretion per g adrenal was significantly larger than after the brief (5 day) treatment with TACE. Long term treatment with TACE led also to hypertrophy of the adrenals, so that the secretion per kg body weight remained unchanged. The adrenals of TACE treated animals showed different degrees of lipid depletion in the zona fasciculata and glomerulosa. Lipid depletion in the zona glomerulosa occurred also in 3 of the 4 control rats. The loss in body weight in the TACE group ranged between 31 and 72 g.

Table 23 lists the experiments in which TACE was dissolved in arachis oil and injected subcutaneously for 15-17 days. Corticosterone secretion amounted to 20.8 µg/g adrenal/minute, whereas that of a control group, injected with arachis oil

TABLE 24

Arachis oil

Cortical secretion of the left adrenal of rats pre-treated with 13 - 15 daily s.c. injections of arachis oil

Date	Rat No.	Strain	No. of injections	b. wt. at start	b. wt. at end	Adrenal plasma (g)	mean body temp. (°C)	l-adrenal mg/kg b. wt.
(m=13) "Normal control group 3" (See Table 5)						mean ± S.E.:		67.7 ± 3.1
23.2 1956	173	B	15	310	258	1.7	33.5	69
23.2 1956	175	B	15	348	270	2.0	37.5	95
15.3 1956	182	B	15	288	262	1.2	35.5	81
15.3 1956	183	B	15	316	298	1.7	35.5	92
14.6 1956	244	O	14	271	284	1.7	37	70
14.6 1956	245	O	14	292	301	0.9	-	60
2.7 1956	265	E	13	290	305	1.5	-	61
2.7 1956	266	E	13	307	325	1.5	-	50
4.10 1956	310	B	15	294	293	1.3	38.5	65
4.10 1956	311	B	15	286	290	1.6	37.5	64
8.10 1956	316	B	15	310	308	1.4	37.5	80
8.10 1956	317	B	15	295	307	1.0	37.5	71
11.10 1956	322	B	15	287	312	2.0	37.5	71
11.10 1956	323	B	15	287	298	1.5	39	75
			mean ± S.E.:					71.7 ± 3.5
Significance of difference from controls:								P > 0.1

TABLE 24 Contd:

(0.8 ml/ kg b.wt.). Blood collected in pentobarbitone anaesthesia over a 15 min. period.

Rat No.	Corticosterone secretion		% re-covery	NaOH fluor-escence	Histology
	µg/g adr./min	µg/hr/adr./kg b.wt.			
	33.1±1.7	135±10.3			
173	34.1	147	75	x:?	normal
175	18.5	108	80	x:+	normal
182	38.4	186	79	x:-	normal
183	38.4	211	70	x:++	normal
244	40.3	170	75	x:++ A:+	very lipid rich gland
245	23.0	83	82	x:++ A:?	very lipid rich gland
265	34.9	128	71	x:+	very lipid rich gland
266	32.9	98	77	x:+	very lipid rich gland
310	32.4	127	71	x:+ A:?	very lipid rich gland
311	23.4	87	62	x:+ A:?	very lipid rich gland
316	25.7	113	78	x:+	very lipid rich gland
317	30.1	128	69	x:+	very lipid rich gland
322	31.8	135	65	x:?	very lipid rich gland
323	25.3	114	62	x:+	very lipid rich gland
	30.7 ± 1.7	131 ± 10			
	P > 0.1	P > 0.1			

only was 30.7 µg/g adrenal/minute (see Table 24). The difference was significant. The hypertrophy of the glands was not as pronounced as in the animals which received TACE orally. The adrenals showed lipid depletion only in the zona fasciculata. It was generally less severe than in the experiments with hexoestrol. The glomerulosa was always filled with lipids. The adrenal function of the oil treated control rats showed no abnormalities, but the lipid content of all three cortical layers appeared to be increased. The oestrogen treated animals lost between 9 and 80 g of their initial body weight, the oil controls showed changes between +18 and -78 g.

Except for rats 169 and 170, which were much heavier than the remaining rats of the group, corticosterone secretion seemed to be larger in rats given TACE in oil than in rats fed TACE in propylene glycol.

If the mean secretion is calculated omitting the two heavy rats secretion per g adrenal per minute becomes 23.1 $\pm$ 1.4 µg, a figure significantly higher than the corresponding figure obtained for rats fed TACE in propylene glycol. Since certain fatty acids have the ability to increase adrenal cholesterol (see p.171), one possible explanation for this difference was interference between the actions of arachis oil and TACE on

TABLE 25

Oestradiol-17β

Cortical secretion of the left adrenal of rats, pretreated with

Group	Blood coll. Date	Rat No.	Strain	Treatment	b.wt.(g) at start	b.wt.(g) at end	adrenal plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b.wt.
	(n=13)		T	"Control group 3" (see Table 5)	mean ± S.E.:				67.7±3.1
1	9.5 1956	199	T	0.8mg oestradiol/kg b.wt/ day, s.c. (0.33% soln. in arachis oil) for 5 days.	265	250	2	35	88
	9.5 1956	200	T		320	287	1.8	38	126
	9.5 1956	201	T		290	268	1.8	35.5	85.8
	11.5 1956	202	T		291	270	1.6	37	80.8
	11.5 1956	203	T		274	254	1.2	38	125.5
	11.5 1956	204	T		313	282	1.2	35	77.4
				mean ± S.E.:					97.3±7.8
	Significance of difference from controls:								P < 0.01
2	20.9 1956	294	T	2mg oestradiol/kg b.wt/ day, s.c. (1% soln. in propylene glycol for 10days	352	303	2.8	34.5	70.3
	20.9 1956	296	T		365	295	2.9	33	60.8
	20.9 1956	297	T		377	332	2.5	39	69.0
				mean ± S.E.:					66.7±3.2
	Significance of difference from controls:								P > 0.1
3	23.7 1956	278	E	2mg oestradiol/kg b.wt/ day, s.c. (1% soln. in propylene glycol) for 5 days	352	330	1.2	36.5	71.2
	23.7 1956	279	O		378	362	2.2	37	89.5
	23.7 1956	280	O		351	340	1.4	37	83.8
	23.7 1956	281	T		356	330	2.3	37.5	87.6

TABLE 25 Contd:

oestradiol-17β. (Blood collected over a 15 min. period in pentobarbitone anaesthesia).

Rat No.	Corticosterone secretion µg/g adr./min.	Corticosterone secretion µg/hr/adr./kg b. wt.	% recovery	NaOH fluorescence	Histology	Remarks
Controls	33.1±1.7	134.7±10.3				
199	30.6	161	86	x:++	some lipid depletion in fasciculata	
200	18.7	141	67	x:++	some lipid depletion in fasciculata	
201	30.0	164	93	x:++	some lipid depletion in outer fasciculata	
202	26.6	133.7	66	x:++	some lipid depleted patches in fasciculata	
203	28.6	215.5	87	x:++	lipid depletion in fasciculata	
204	20.2	93.6	79	x:++	some lipid depleted patches in fasciculata	
	25.8±1.9	151.5±19.7				
	P=0.05-0.02	P > 0.1				
294	27.6	116.0	83	x:+	normal	jaundice
296	19.4	70.5	75	x:?	lipid depleted patches in fasciculata	
297	24.6	102.0	73	x:?	lipid depleted patches in fasciculata	slight jaundice
	23.9±2.8	96.2±15.5				
	P = 0.05	P > 0.05				
278	58.4	249	69	x:++ A:+	(laden all layers lipid	
279	29.6	157	78	x:+ A:?	some lipid depletion in fasciculata	
280	20.3	102	65	x:?	severe lipid depletion in fasciuclata	slight jaundice
281	31.5	166	70	x:?	some lipid depletion in fasciculata	

steroid metabolism. In order to check on this possibility two groups of rats were treated with a combination of oral administrations of TACE and subcutaneous injections of arachis oil, one for 5, the other one for 15 days. In both groups, the mean figures for corticosterone secretion did not differ significantly from those of rats fed TACE alone (see Tables 21 and 23). Any difference attributed to the oil in the previous experiments could, therefore, only be caused by less effective absorption of the TACE from the oil solution.

Oestradiol-17β

Details of the results obtained in experiments with the naturally occurring oestrogen oestradiol-17β are given in Tables 25 and 26.

Oestradiol-17β was on the whole, less active in inhibiting adrenocortical secretion than the synthetic oestrogens. The individual variations were large. When administered in greater quantities, the rats showed signs of severe liver damage.

When rats were treated with 5 daily injections of 0.8 mg oestradiol/kg (in arachis oil), their left adrenals secreted a mean of 25.8 ± 1.9 μg corticosterone/g adrenal/minute (Table 25, Group I. This figure is only 22% smaller than the mean

TABLE 26

Oestradiol 17-β

Cortical secretion of the left adrenal of control rats pretreated with 5 daily injections of propylene glycol and of rats, pretreated with 5 daily

Group	Date	Rat No.	Strain	Treatment	b.wt. (g) at start	b.wt. (g) at end	adrenal plasma (g)	Mean body temp. (°C)	l. adrenal mg/kg b.wt.
4	28.1 1957	350	0	0.2 ml propylene glycol/ kg/day, s.c. for 5 days	302	318	2.4	37	100
	28.1 1957	351	0		284	298	2.4	38	76
	28.1 1957	352	0		279	290	1.9	37.5	70
	31.1 1957	356	0		326	342	2	37.5	59
	31.1 1957	358	0		297	312	1.9	38	58
	mean ± S.E.:								72.6 ± 8
5	28.1 1957	347	0	2 mg oestradiol/kg b.wt/ day, s.c. (1% soln. in propylene glycol) for 5 days	307	283	1.9	33	80
	28.1 1957	348	0		287	255	1.6	36	84
	28.1 1957	349	0		308	276	2.6	34.5	92
	31.1 1957	353	0		318	301	1.5	36	93
	31.1 1957	354	0		308	280	1.6	38	104
	mean ± S.E.:								90.6 ± 4.6
	Significance of difference from controls:								P > 0.05

TABLE 26 Contd:

injections of oestradiol 17-β (blood collected over a 15 min. period in pentobarbitone anaesthesia).

Rat no.	Corticosterone secretion µg/g adr./min	µg/hr/adr./kg. b. wt.	% re-covery	NaOH fluor-escence	Histology
350	28.3	169	77	x:+++	some lipid depletion in fasciculata
351	30.2	137	64	x:+++	normal
352	29.7	124	65	x:++	normal
356	27.3	96	69	x:+	normal
358	31.1	108	90	x:+	normal
	29.3±0.73	127 ± 14			
347	16.8	81	69	x:+	lipid depletion in fasciculata
348	24.0	121	79	x:+	severe lipid depletion in fasciculata
349	16.8	93	76	x:++	severe lipid depletion in fasciculata
353	22.8	149	56	x:+	severe lipid depletion in fasciculata
354	29.7	185	75	x:+	slight lipid depletion in fasciculata
	22.0±2.48	126 ± 17.7			
	P=0.02-0.01	P > 0.1			

secretion of the corresponding control group. The adrenals were enlarged, therefore this represented no decrease in the secretion/kg body weight. All chromatograms showed clear spots in the x-region. The histological pictures of the adrenals were little changed. Only a mild degree of lipid depletion could be observed. There was a ring of enlarged vessels between inner and outer fasciculata. The rats lost 15 to 33 g weight during the treatment.

The next experiment (Group 5, Table 26) was done with a larger dose dissolved in propylene glycol. The O- strain was used. The adrenal function of rats treated with 5 daily injections of 2 mg oestradiol/kg was moderately inhibited. The mean corticosterone secretion rate was 22.0 ± 2.5 µg/g adrenal/minute. It was 25% lower than that of the corresponding control rats treated with 5 daily injections of propylene glycol only. Again, the amount of corticosterone secreted per kg body weight was not decreased. Adrenal lipid depletion was more pronounced than in the rats which received the smaller dose. There was also a slight lipid loss in the adrenals of the control rats. Loss in body weight ranged between 17 and 32 g. All control rats gained weight during the injection period.

Seven rats from other strains (see Table 25,

Group 3) had been treated at an earlier date in the same fashion. They responded very irregularly. In one of them (No.278) the secretory capacity of the adrenal was found far above the normal range, and all three layers of its adrenal cortex were lipid laden; another rat (No.280) had a low secretion rate, severe lipid depletion in the gland and was jaundiced.

When daily injections of 2 mg oestradiol-17β (in propylene glycol) were continued for 10 days, (Table 25, Group 2), the general state of health of the animals was severely reduced. They lost between 43 and 70 g weight, were jaundiced and operation was difficult because of great fragility of the blood vessels. Despite that, the secretory capacity of the adrenal was seriously inhibited only in one rat (No.296).

The calculated means for the corticosterone secretion/g adrenal/minute for the different groups of oestradiol treated rats were in each instance lower than that of the corresponding control group. The value for P was however, never as low as 0.01. In no case a significant decrease in the secretion per kg body weight occurred. The hypertrophy of the adrenals was never very pronounced.

Discussion

The foregoing results show that the inhibitory action of hexoestrol on the hormone secretion of the rat adrenal cortex, observed under conditions of stress, is not an action peculiar to this oestrogen. Three other substances with oestrogenic properties, chlorotrianisene, ethinyloestradiol and, to a lesser degree, the natural hormone oestradiol-17β, produce a similar effect.

An inhibition of adrenocortical secretion by chlorotrianisene was observed in several groups of rats, treated with the drug in different ways. In all animals, the amount of corticosterone secreted per g adrenal tissue was markedly decreased, impairment of adrenal function being less pronounced with increasing duration of treatment. After prolonged pretreatment the adrenals became sufficiently enlarged to compensate for the lower secretory capacity per g tissue.

Ethinyloestradiol consistently reduced the secretory capacity of the adrenal cortex per g gland tissue and also per kg body weight.

The inhibitory effect of the naturally occurring oestrogen oestradiol-17β on corticosterone secretion of rat adrenals was very variable. In each group of experimental animals some rats res-

ponded with a marked decrease in adrenal secretion rate, whereas in others the adrenals did not undergo any functional or structural changes.

Whenever the corticosterone secretion of an adrenal was found to be diminished as a consequence of the administration of an oestrogen, a corresponding decrease in the secretion of Bush's compound X could be observed. At the same time, the sudanophilic lipids of the zona fasciculata were reduced, or had disappeared.

The quantities of the different oestrogens sufficient to produce structural changes and functional inhibition of the adrenal cortex after daily administrations were 0.4 mg of hexoestrol, 40 mg of TACE, 2 mg of ethinyloestradiol and 2 mg of oestradiol-17β per kg body weight. In the vaginal cornification test on the castrated female rat hexoestrol was found to be 100 times more active than TACE, (Thompson and Werner, 1945), and approximately three times more active than oestrone (Campbell et al., 1939). Since oestradiol-17β is five to ten times more active than oestrone in the rat (see Pincus, 1948), its oestrogenic potency is only slightly higher than that of hexoestrol.

The oestrogenic potency of subcutaneously injected ethinyloestradiol is approximately equal to that of oestradiol-17β in this test (Inhoffen and

Hohlweg, 1938a, b). Thus, on the basis of their oestrogenic potencies, hexoestrol and TACE are about equal, and ethinyloestradiol about five times less effective in impairing adrenocortical function. Oestradiol was less effective than ethinyloestradiol, but no quantitative data were obtainable since the doses used were the highest compatible with survival of the animals.

The inhibitory action of hexoestrol on rat adrenals has tentatively been explained as an interference with cholesterol synthesis (Vogt, 1955), as it is generally assumed the cholesterol is the main precursor of adrenal steroids.

Strong support for this theory was obtained by Boyd and McGuire (1956), who studied the in vitro synthesis of cholesterol from C^{14}-acetate by liver slices of normal rats and of rats pretreated with hexoestrol. When 0.8 mg/kg were injected daily for six days, cholesterol synthesis was found to be considerably diminished. So was the cholesterol concentration in serum and adrenal glands. Similar studies have been carried out with ethinyloestradiol and oestradiol-17β. Rats pretreated with 0.25 - 2.5 mg ethinyloestradiol per kg per day for 11-26 days, showed a tendency towards reduction of both serum and adrenal cholesterol. The rate of hepatic synthesis of cholesterol from C^{14}-acetate in vitro by liver slices of these rats was, however,

only "inconsistently affected" (McGuire, 1956). The effect of oestradiol-17β on cholesterol synthesis has only been studied in two rats in which the hepatic synthesis of cholesterol from C^{14}-acetate in vitro was found to be reduced by 50%. The rats were given first 1.9 and later 3.9 mg/kg/day for a month. This treatment did not affect the cholesterol concentration in serum or adrenals (McGuire, 1955). No studies on the cholesterol metabolism of rats pretreated with TACE are reported in the literature.

Although the foregoing studies on the effect of ethinyloestradiol and oestradiol-17β on cholesterol metabolism do not yet give clear information, they indicate a tendency of these compounds to impair cholesterol synthesis. This makes it likely, that their action on adrenocortical function involves a similar mechanism as the action of hexoestrol.

In order to obtain a more complete understanding of the overall significance of the effect of oestrogens on the adrenal cortex, at least three more questions have to be answered:

1. How much corticosterone is secreted by the adrenal cortex of an oestrogen pretreated rat under "resting conditions"?

2. Do oestrogens also interfere with the syn-

thesis and secretion of aldosterone?

3. Is this effect of oestrogens peculiar to the rat or is it also produced in other mammalian species?

1. Thus far the attempts to answer the first question have failed, since it has not been possible to obtain adrenal vein blood from a rat under resting conditions (see p. 182). Some information might be obtained by indirect methods, for example, by estimating the corticosterone content of adrenals of oestrogen pretreated rats, obtained after rapid decapitation of the animal. There are indications that the resting secretion might be higher than in normal animals. Thus administration of hexoestrol causes involution of the thymus, which is absent in adrenalectomised rats (Brolin and Hellman, 1953) and might be attributed to increased corticosterone secretion.

Figures for the ACTH concentration in peripheral plasma of rats treated with oestradiol amount to 9m.u/100 ml according to Gemzell (1952). Sydnor and Sayers (1953) obtained similar figures (2.8 - 8 m.u./100 ml) for rats slowly exsanguinated in ether anaesthesia. If these figures, which were obtained with different methods, are comparable, the corticosterone secretion of a

hexoestrol treated rat may already be maximal before the operative stress is applied.

2. To speculate on the second question on basis of our present knowledge is of doubtful value. It is however, by no means certain that aldosterone secretion will be interfered with to a similar extent. For example, the lipid content of the zona glomerulosa, where aldosterone is synthesised (Giroud et al. 1956), is hardly affected by 4-6 daily injections of an oestrogen. The observation that ACTH does but little influence aldosterone secretion in the rat (Singer and Stack-Dunne, 1955) is another sign of the special position of aldosterone amongst adrenal steroids. Experiments for the determination of the amount of aldosterone secreted into adrenal vein blood in hexoestrol treated rats are planned.

3. Investigations on the effect of oestrogens on adrenals of mammalian species other than rats have been carried out by Vogt (1955) on rabbits. In these animals, no consistent structural or functional changes in the adrenals were observed after treatment with very large doses of hexoestrol. Contrary to rats, rabbits respond to hexoestrol with a hypercholesterolaemia (McGuire, 1956).

Adrenocortical enlargement was observed in mice after implantation of oestrone and oestradiol (Deansely, 1953). Administration of 200 µg oestradiol per day to male and female monkeys (Macacus ecaudatus) for a month increased the lipid content of the zona fasciculata. The cell nuclei appeared pyknotic (Guyon and Marois, 1954).

Whether oestrogens produce an effect on human adrenals is not yet established. Depression of plasma total cholesterol and of the cholesterol attached to the β-lipoproteins was observed in man after administration of ethinyloestradiol. Administration of hexoestrol resulted in a similar trend, but the effect was less pronounced (Oliver and Boyd, 1956).

Summary

1. Groups of rats were treated with hexoestrol, chlorotrianisene, ethinyloestradiol and oestradiol-17β. Sixteen to twenty-four hours after the last drug administration adrenal vein blood was collected under conditions of operative stress and its corticosterone content determined.

2. Compared on the basis of their oestrogenic potencies the doses required to obtain an effect were equal for hexoestrol and chlorotrianisene, five times higher for ethinyloestradiol and oestra-

diol-17β.

3. The amount of corticosterone secreted per g adrenal after pretreatment with hexoestrol, chlorotrianisene and ethinyloestradiol was found to be significantly reduced.

4. With the natural hormone oestradiol-17β such inhibition was less constantly seen.

5. Reduced corticosterone secretion was regularly associated with reduced lipid content of the zona fasciculata.

6. Interference with cholesterol synthesis as the possible mechanism of action is discussed.

CHAPTER 8

Restoration of normal secretion in a hexoestrol inhibited rat adrenal

In the foregoing chapter it has been described that in the rat oestrogens exert a depressing effect on adrenocortical secretion measured under conditions of operative stress. It was suggested that this effect is due to interference of oestrogens with cholesterol synthesis.

The experiments described in this chapter have been carried out in order to investigate whether the depressing effect of hexoestrol on corticosterone secretion can be antagonized on the basis of this theory. The measures taken were calculated to increase cholesterol concentration either in the blood supplying the adrenal gland or in the adrenal gland itself.

Methods

Attempts were made to overcome the effect of hexoestrol either by infusing substances intravenously in hexoestrol treated rats before collecting a sample of adrenal vein blood, or by simultaneous pretreatment of rats with hexoestrol and

substances known or supposed to increase the adrenal cholesterol concentration.

Adrenal vein blood was collected under pentobarbitone anaesthesia as described in Chapter 2. In most experiments designed to test the effect of intravenous infusions, two adrenal blood samples were collected, separated by a 10 min. interval. In such animals, the entry of the renal vein into the vena cava was occluded by a clip instead of a ligature. During the interval this clip was removed and the collecting tube occluded. A first control sample was collected for 15 min, followed by a 10 min. interval during which 4 ml of the solution in question were infused. Then collection of a second sample was started while another 4 ml were infused for 10 min. After the infusion was stopped adrenal blood was collected for an additional 5 min. to complete the 15 min. period.

In the animals pretreated simultaneously with hexoestrol and the substance under investigation, only one adrenal vein blood sample was collected in the usual way.

The corticosterone content in adrenal vein blood was estimated by the method described in Chapter 2.

1. Intravenous infusions

Experiments were done on adult male rats. All rats were pretreated with 4-15 daily subcutaneous injections of 0.4 mg hexoestrol per kg body weight. Infusion and blood collection were carried out 16-24 hours after the last injection.

a. Normal rat plasma: Plasma was obtained from large rats by cannulating one carotid artery (see p. //). The red cells were centrifuged off. Nine hexoestrol pretreated rats (B- and T- strain) received intravenous infusions of 8 ml normal rat plasma (Table 27).

b. Plasma from rats pretreated with hexoestrol: (5-6 daily injections of 0.4 mg/kg b. wt.) was obtained in the same fashion. Infusions were done in 6 rats (B- strain), (Table 28).

c. Albumin: 5% bovine albumin solutions were made from a 30% Armour and a 20% Behring preparation by dilution with the requisite amount of sodium chloride in water to assure isotonicity. Eight rats (B- strain) received infusions of these solutions (Table 29).

d. Progesterone was added either to plasma from hexoestrol treated donor rats or to a 5% bovine albumin solution. A 1.1% solution of pro-

gesterone in propylene glycol was added slowly to the vigorously stirred plasma or albumin and the clear mixture infused. Each rat received 1.3 mg progesterone in 8 ml vehicle. Experiments were carried out on 5 groups of rats. Four groups consisted of hexoestrol pretreated animals. The rats of group 1, 2 and 4 received intravenous infusions of progesterone, dissolved in plasma from hexoestrol treated rats, albumin or normal rat plasma. In the rats of group 3, progesterone was infused into the aorta. A 2.2% solution of progesterone in propylene glycol was added under stirring to 5% bovine albumin, progesterone concentration and total dose infused being double that given by the intravenous route. A fine syringe needle was connected to the infusion apparatus and inserted into the aorta at the level of the diaphragm. There was no bleeding from the puncture (Table 30).

e. α-lipoprotein. It consisted of a human plasma fraction IV-1 (Cohn et al. 1946) of which 0.25 g were ground up with 10 ml 0.9% NaCl solution, shaken mechanically for 70 minutes and centrifuged. 8 ml of the supernatant were infused. Contrary to expectation the supernatant only contained 35 mg cholesterol per 100 ml. Most of the cholesterol

remained in the bottom paste (Table 31).

f. β-lipoprotein. It was prepared by Dr. Boyd from human plasma fraction III-0 by the method of Oncley et al. (1950). Before use it was spun in a refrigerated centrifuge at 5°C. The supernatant which contained 310 mg cholesterol per 100 ml was either used undiluted or as a 1 in 4 dilution in phosphate buffer of pH 7.6 (Table 31).

g. Cholesterol. A colloidal cholesterol solution was prepared by the method of Byers and Friedman (1949). 1.5 g cholesterol were dissolved in 25 ml ether and added under constant stirring to 50 ml of a 1% solution of lauric acid in 0.05N NaOH. The mixture was passed several times through a glass homogenizer of the cylinder and piston type and centrifuged. It was either infused intravenously immediately before collection of a blood sample or injected intravenously (tail or hind paw) 2 to 3 hours before blood collection.

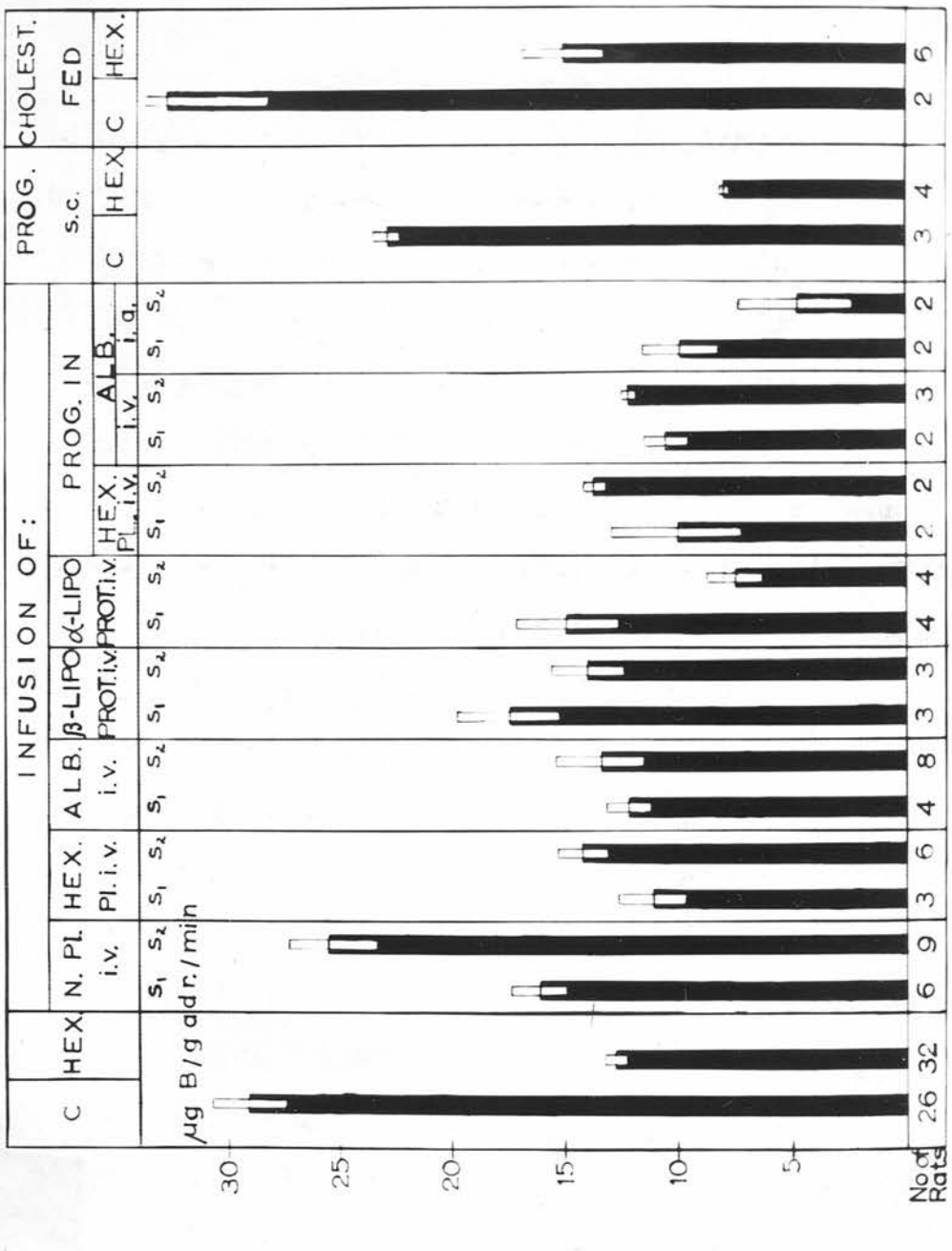
2. Long lasting pretreatment

a. Progesterone. For subcutaneous injections a 2% solution in arachis oil was used. Injections were made 24 and 2 hours before the collection of adrenal vein blood. The dose at each injection was either 20 or 25 mg/kg (Table 32).

b. Cholesterol was administered to rats by feeding a paste of ground rat cake containing 1.4% (w/w) cholesterol and 8.6% (w/w) olive oil. The cholesterol was dissolved in hot olive oil and 10g of the solution mixed with 90 g dry, ground cake. Water was added immediately before use to make a paste. Eight rats (B- strain) were fed in this fashion for 14 to 17 days. Six of them received daily injections of hexoestrol (0.4 mg/kg/day) for the last five days of the cholesterol rich diet, 2 served as controls (Table 33).

c. Erucic acid was administered to rats by feeding a paste of ground rat cake containing 10% or 12% (w/w) erucic acid. Water was added immediately before feeding to make a paste. Fourteen rats were fed on this diet for 14 to 28 days. Six of them (E- and T- strain) received erucic acid only (Table 33a), 8 (B- strain) in addition 5 daily injections of hexoestrol (0.4 mg/kg/day) immediately prior to the day of blood collection (Table 33b). As the food intake decreases during treatment with hexoestrol, 4 g erucic acid emulsed in 6 ml warm milk were fed to each rat by stomach tube daily on the last 2 days of hexoestrol treatment.

Figure XIII



Effect of short infusions or repeated administrations of different substances on the corticosterone secretion of hexoestrol inhibited rat adrenals.
C = Controls. Hex. = Hexoestrol injected rats. N. Pl. = Normal rat's plasma. Hex. Pl. = Plasma of hexoestrol treated rats. Alb. = albumin. Prog. = Progesterone.

S_1 before S_2 during infusion

Results

Fig. XIII gives a survey on the results obtained.

1. Intravenous infusions

a. Normal rat plasma. Infusion of 8 ml normal rat plasma into hexoestrol pretreated rats resulted in a striking increase of the secretory capacity of the adrenal. The details of the experiment are listed in Table 27. All rats responded to this infusion with an increased output of corticosterone, though the degree of the restoration varied. The effect tended to be larger when the initial secretion rate had been more depressed.

b. Plasma from rats pretreated with hexoestrol. Control experiments were performed in which the rats used as plasma donors were themselves pretreated with hexoestrol. Again, 8 ml plasma were infused into the experimental animals. Such an infusion did not significantly improve adrenocortical secretion rate (Table 28). Blood flow and blood pressure were changed equally in both experiments. Thus it was likely that there was a substance present in normal rat plasma and lacking in plasma from hexoestrol treated rats, which was responsible for the beneficial effect of normal rat plasma.

TABLE 27

Infusion of normal rat plasma

The effect of an i.v. infusion of 8 ml normal rat plasma on the corticosterone secretion of the left adrenal of hexoestrol treated rats. The plasma was infused 10 min. prior to and during the first 10 min. of collection of S_2. In 6 rats, a control sample S_1 was collected during

Date	Rat No.	Strain	No. of hex. inj.	b. wt. (g) at start	b. wt. (g) at end	mean body temp. (°C) S_1	mean body temp. (°C) S_2	mean blood pressure (mm Hg) S_1	mean blood pressure (mm Hg) S_2	adrenal plasma (g) S_1	adrenal plasma (g) S_2
13.6 1955	98	B	5	300	255	-	-	-	105	-	3.8
13.6 1955	99	B	5	295	250	-	-	-	120	-	3.1
13.6 1955	100	B	5	307	266	-	38	-	140	-	5.0
22.6 1955	106	B	5	390	357	37	37	100	90	2.2	4.0
3.5 1956	193	T	4	310	270	39	37	160	130	1.4	1.7
3.5 1956	194	T	4	298	264	33.5	34	100	150	1.5	4.8
14.5 1956	207	T	4	270	255	36	35	-	-	2.6	5.7
14.5 1956	208	T	4	250	251	35	37	-	-	1.7	4.3
31.5 1956	232	B	15	376	313	38	37	120	120	1.7	3.2
				mean (Nos. 106, 193, 194, 207, 208, 232) ± S.E.:							
				Significance of difference between S_1 and S_2:							

TABLE 27 Contd:

the 15 min. period preceding the plasma infusion. (Pentobarbitone anaesthesia, all samples collected for 15 min.)

Rat No.	% recovery		NaOH-fluorescence		B-secretion: µg/g adr./min.		% change
	S_1	S_2	S_1	S_2	S_1	S_2	
98	-	72	-	x:++	-	25.1	-
99	-	101	-	x:+	-	19.3	-
100	-	81	-	x:?	-	27.4	-
106	72	72	x:+	x:+	14.1	23.1	+63.5
193	82	82	x:+	x:+++	19.6	22.1	+13
194	70	74	x:?	x:+++	18.0	23.4	+30
207	65	70	x:++	x:++	19.8	33.3	+68
208	64	75	x:?	x:+++	11.9	25.5	+114
232	84	82	-	-	13.2	25.6	+94
					16.1 ± 1.3	25.5 ± 1.8 (last six rats)	

$P < 0.01$

TABLE 28

Infusions of plasma from hexoestrol treated rats

The effect of an i.v. infusion of 8 ml plasma from hexoestrol treated rats (B-strain), (5 daily injections) on the corticosterone secretion of the left adrenal of hexoestrol treated rats. The plasma was infused at a speed

Date	Rat No.	No. of hex. inj.	b. wt. (g)		mean body temp. (°C)		mean blood pressure (mm Hg)		adrenal plasma (g)	
			at start	at end	S_1	S_2	S_1	S_2	S_1	S_2
20.6 1955	102	5	321	262	-	38	-	153	-	2.7
20.6 1955	103	5	312	264	-	39.5	-	120	-	4.5
22.6 1955	105	5	341	295	-	-	-	125	-	4.8
27.6 1955	107	5	325	293	35.5	-	110	100	2	6
30.6 1955	108	5	320	262	36.5	37	110	110	0.9	2.9
30.6 1955	109	5	362	336	39.0	-	80	60	1.3	3.8
					mean ± S.E.:					
					Significance of difference between S_1 and S_2					

TABLE 28: Contd:

of 0.4 ml/min. for 10 min. prior to and for 10 min. during the collection of S_2. In 3 rats, a control sample S_1 was collected before the infusion. (Pentobarbitone anaesthesia, all samples collected for 15 min.)

Rat No.	% recovery		NaOH fluorescence		B-secretion: µg/g adr./min.		% change
	S_1	S_2	S_1	S_2	S_1	S_2	
102	-	68	-	x:+	-	13.5	-
103	-	95	-	x:+	-	17.7	-
105	-	77	-	x:-	-	11.0	-
107	75	75	-	-	10.8	15.0	+13.9
108	72	72	x:+	x:+	9.0	13.2	+14.5
109	72	72	x:+	x:+	13.4	14.9	+11.1
					11.1±1.5	14.2±1.1	+13.1
					P > 0.1		

TABLE 29

Albumin infusions

The effect of an intravenous infusion of 8 ml bovine albumin (5% solution in 0.9% NaCl) on the corticosterone secretion of the left adrenal of hexoestrol treated rats. The plasma was infused at a speed of 0.4 ml/min. for 10 min. prior to and 10 min. during the collection of S_2. In 4 rats, a control sample S_1 was collected

Date of blood coll.	Rat No.	b.wt.(g) at start	b.wt.(g) at end	No. of hex. inj.	Mean body temp.(°C) S_1	Mean body temp.(°C) S_2	Mean blood pressure (mm Hg) S_1	Mean blood pressure (mm Hg) S_2	plasma S_1	plasma S_2
4.1 1956	135	418	356	6	-	-	-	85	-	5.8
4.1 1956	137	376	324	6	-	-	-	120	-	3.2
10.1 1956	140	360	333	5	-	-	-	130	-	7.3
10.1 1956	141	368	316	5	-	34	-	-	-	4.7
10.1 1956	138	428	400	5	36	-	120	120	2	4.2
12.1 1956	142	406	357	5	38	38.3	120	120	2.1	3.4
12.1 1956	143	358	332	5	-	-	110	95	2.3	5.5
16.1 1956	144	333	290	9	-	-	120	70	2.6	5.2
				mean ± S.E.:						
Significance of difference between S_1 and S_2										

TABLE 29 Contd:

before the infusion. (Pentobarbitone anaesthesia, all samples collected for 15 min.)

Rat No.	% recovery		NaOH-fluorescence		B-secretion: µg/g adr. min.		% change
	S_1	S_2	S_1	S_2	S_1	S_2	
135	-	59	-	x:++	-	13.3	-
137	-	60	-	x:+	-	25.8	-
140	-	63	-	x:+	-	10.2	-
141	-	65	-	x:-	-	12.7	-
138	61	61	x:+	x:+	13.2	10.0	-24.3
142	61	64	x:-	x:-	12.8	11.5	-10.2
143	76	67	x:+	x:+	9.3	9.5	+2.0
144	65	68	x:+	x:+	13.6	14.4	+6.0
					12.2 ± 1.0	13.4 ± 2.0	
					$P > 0.1$		

c. Albumin. Another series of control experiments was carried out by infusing albumin solutions into hexoestrol pretreated rats. These experiments were done in order to rule out the possibility that the beneficial effect of normal rat's plasma was due to a dilution of the hexoestrol present in the tissues by the large volume of hexoestrol-free fluid infused. The results are given in Table 29. They clearly show that, although the rise in blood pressure and blood flow caused by albumin infusions, was the same as after infusions of normal plasma, no increase in cortical secretion was obtained.

The only known difference between plasma of normal and plasma of hexoestrol pretreated rats, is their cholesterol content. The plasma concentration of total cholesterol falls during hexoestrol treatment by about 90% (Boyd and McGuire, 1956). The simplest explanation for the beneficial effect of an infusion of normal rat plasma in hexoestrol pretreated rats would therefore be, that it supplies the adrenal cortex with sufficient amounts of cholesterol, from which it can synthesize its hormones. Final proof that no other substance is involved would require the demonstration that cholesterol added to a vehicle from which it it can be taken up by tissues also re-

stores adrenal function.

It is very difficult to produce artificially a combination of protein and cholesterol which imitates the lipoprotein-cholesterol complex present in plasma.

The following attempts were made to offer large amounts of cholesterol to a cholesterol depleted adrenal in an acute experiment.

d. Colloidal cholesterol solution. A colloidal solution of cholesterol in sodium laurate was either injected intravenously into conscious rats, or infused anaesthetized rats, after one adrenal blood sample had been collected. When infused in the doses recommended by Byers and Friedman the animals died of pulmonary oedema, whether pretreated with hexoestrol or not. When doses of 0.5 ml/rat were injected into conscious rats, these showed signs of respiratory embarrassment and brain damage, but 3 rats survived the injection and approximately 3 hours later an adrenal vein blood sample was collected in ether anaesthesia. The corticosterone secretion was severely depressed, in spite of the fact that blood pressure and adrenal blood flow were normal during the collection period. Damage of the brain could perhaps have been the cause of a fall in blood ACTH.

TABLE 31

Infusion of human α- or β-lipoproteins. The effect of an i.v. infusion of α- or β-lipoproteins on the corticosterone secretion of the left adrenal of hexoestrol treated rats.

GROUP 1: After the collection of a control sample (S_1) 8 ml of a suspension of human β-lipoprotein containing per 100 ml, 310 mg cholesterol were infused at a speed of 0.4 ml/min. either undiluted (rats No.163 and 164) or as a 1:4 dilution in phosphate buffer of pH 7.6 (rat No.162). S_2 was collected during the second half of the infusion and the following 5 min.

GROUP 2: After the collection of a control sample (S_1) 8 ml of a suspension of human α-lipoproteins containing per 100 ml 35 mg cholesterol were

Blood coll. Date	Rat No.	b.wt. (g) at start	b.wt. (g) at end	No. of Hex. inj.	Infusion	mean body temp. (°C) S_1	mean body temp. (°C) S_2	mean blood pressure S_1	mean blood pressure S_2	plasma (g) S_1	plasma (g) S_2
13.2 1956	163	430	365	5	β-lipoprotein undiluted	35.5	36	114	95	2.5	2
13.2 1956	164	401	344	5	β-lipoprotein undiluted	37.5	"	135	130	1.2	0.9
13.2 1956	162	358	313	5	β-lipoprotein; 1:4 dilution	34.5	"	100	80	1.7	2.1
					mean ± S.E.:						
23.1 1956	145	403	353	5	α-lipoprotein	36.2	"	70	45	2.5	2.4
23.1 1956	146	368	323	5	α-lipoprotein	37.5	"	125	80	1.4	1.5
23.1 1956	147	347	310	5	α-lipoprotein	38.5	"	-	-	1.2	2.5
26.1 1956	151	365	300	6	α-lipoprotein	35	34.5	120	100	1.9	2.3
					mean ± S.E.:						

TABLE 31

infused. S_2 was collected during the second half of the infusion and the following 15 min. (Pentobarbitone anaesthesia, all samples collected for 15 min.)

Rat No.	% recovery		NaOH-fluorescence		B-secretion: µg/g adr./min.		% change
	S_1	S_2	S_1	S_2	S_1	S_2	
163	69	84	x:-	x:+	14.1	12.2	-13.5
164	68	84	x:+	x:?	20.9	17.0	-18.6
162	72	-	x:+	x:+	17.6	12.7	-27.8
					17.5±2.3	14.0±1.6	
145	72	63	x:++	x:+	19.2	9.4	-51
146	75	76	x:+++	x:-	15.0	9.5	-36.7
147	66	70	x:-	x:-	15.7	4.6	-70.7
151	62	64	x:-	x:-	9.8	6.7	-31.6
					14.9±2.3	7.5±1.2	

Experiments were then performed in which human lipoproteins were infused.

e. Human α-lipoproteins. A solution of α-lipoproteins, the form in which cholesterol is carried in the rat, was mixed with a 0.9% NaCl solution, the precipitate centrifuged off and the supernatant infused. As can be seen in Table 31, the infusions caused severe depression of corticosterone secretion. They also failed to raise the blood pressure as did infusions of plasma or albumin. The cholesterol concentration of the solution infused was only 35 mg/100 ml.

f. Human β-lipoproteins. The next trial was done with a freshly prepared preparation of β-lipoprotein, which contained 310 mg cholesterol per 100 ml. This is far higher than the cholesterol concentration in normal rat plasma, which is approximately 65 mg/100 ml. Nevertheless, no increase in the secretion rate was obtained (see Table 31), nor did the blood pressure rise.

The fact that neither the lipoprotein solutions nor the cholesterol emulsions were well tolerated suggests that the particle seize was so large as to cause haemagglutination and to cause failure of the blood circulation. A similar phenomenon was

TABLE 30

Progesterone infusions

Corticosterone secretion of normal and hexoestrol treated rats after infusion of progesterone solutions.

Group 1: Hexoestrol rats - i.v. infusion of 1.3 mg progesterone in 8 ml plasma of hexoestrol treated rats.

Group 2: Hexoestrol rats - i.v. infusion of 1.3 mg progesterone in 8 ml bovine albumin (5% in 0.9% NaCl).

Group 3: Hexoestrol rats - infusion into the aorta of 2.6 mg progesterone in 8 ml bovine albumin (5% in 0.9% NaCl)

Date	Rat No.	b. wt. (g) at start	b. wt. (g) at end	No. of Hex. inj.	Infusion	mean body temp. (°C) S_1	mean body temp. (°C) S_2	mean blood pressure S_1	mean blood pressure S_2	plasma (g) S_1	plasma (g) S_2
4.7 1955	111	367	305	4	Group 1: Progest. in plasma of hex. treated rats i.v.	38.5	38	110	120	1.5	4.5
4.7 1955	112	358	301	4		39	38	110	110	1.3	4.5
					mean ± S.E.:						
26.1 1956	148	320	253	6	Group 2: Progest. in bovine albumin i.v.	-	38	100	120	1.1	2.5
26.1 1956	149	325	252	6		33	34.5	80	90	3.3	5.7
26.1 1956	150	341	282	6		-	37	-	120	-	2.5
					mean ± S.E.:						
9.2 1956	160	310	247	5	Group 3: Progesterone in bovine albumin into the aorta	38	38	120	40	0.8	0.7
9.2 1956	161	400	340	5		32.5	34	100	100	2.8	1.4
					mean ± S.E.:						

CONTD:

TABLE 30 Contd:

Group 4: Hexoestrol rats - i.v. infusion of 1.3 mg progesterone in 8 ml plasma of normal rats.

Group 5: Normal rats - i.v. infusion of 1.3 mg progesterone in 8 ml plasma of normal rats.

All solutions were infused at a speed of 0.4 ml/min, 10 min. prior to and during the first 10 min. of the collection of S_2. In some animals a control sample S_1 was collected before the infusion. (Pentobarbitone anaesthesia, all samples collected for 15 min.)

Rat No.	% recovery		NaOH-fluorescence		B-secretion: µg/g adr./min.		% change
	S_1	S_2	S_1	S_2	S_1	S_2	
111	85	78	x:-	x:?	11.9	14.1	+18.5
112	62	65	-	-	7.2	13.2	+83.0
					10.0±2.9	13.7±0.56	
148	60	59	x:+	x:+	11.3	12.4	+11.1
149	69	64	x:+	x:+	9.9	11.7	+18
150	-	66	-	-	-	12.6	-
					10.6±0.88	12.2±0.3	
160	63	65	x:-	x:-	8.2	2.3	-72
161	lo-st	75	x:+	x:-	11.6	7.4	-
					9.9±1.7	4.8±2.5	

CONTD:

TABLE 30 CONTD:

Date	Rat No.	b.wt. (g) at start	b.wt. (g) at end	No. of Hex. inj.	Infusion	mean body temp. (°C) S_1	mean body temp. (°C) S_2	mean blood pressure S_1	mean blood pressure S_2	plasma (g) S_1	plasma (g) S_2
6.6 1955	94	372	360	4		-	-	-	140	-	5.5
6.6 1955	95	270	245	4	Group 4: Progesterone in normal rat plasma i.v.	-	-	-	85	-	2.9
8.6 1955	96	298	265	6		-	-	-	120	-	5.2
8.6 1955	97	323	305	6		-	39	-	150	-	3.8
					mean ± S.E.:						
Difference from rats treated with hexoestrol only (see Table 19)											
26.5 1955	90	-	360	0		-	39	-	-	-	2.6
31.5 1955	91	-	285	0	Group 5: Progesterone in normal rat plasma i.v.	-	36	-	120	-	5.0
31.5 1955	92	-	330	0		-	-	-	130	-	3.3
					mean ± S.E.:						

TABLE 30 CONTD:

Rat No.	% recovery		NaOH-fluorescence		B-secretion: µg/g adr./min.		% change
	S_1	S_2	S_1	S_2	S_1	S_2	
94	-	91	-	-	-	19.6	-
95	-	69	-	-	-	18.0	-
96	-	82	-	-	-	24.8	-
97	-	65	-	-	-	28.9	-
						22.8 ± 2.6	
						$P < 0.01$	
90	-	-	-	x:-	-	18.4	-
91	-	84	-	x:+	-	29.6	-
92	-	75	-	x:+	-	22.2	-
						23.3 ± 3.8	

TABLE 32

Progesterone, subcutaneous injections

Corticosterone secreted by the left adrenal of normal and of hexoestrol treated rats. All rats received subcutaneous injections of pro-

Date	Rat No.	Strain	No. of: Hex. inj.	No. of: Progest. inj.	b. wt. (g) at start	b. wt. (g) at end	plasma (g)	Mean body temp. (°C)
21.4 1955	83	B	0	2 x 20 mg/kg	.	325	1.8	-
21.4 1955	84	B	0	2 x 20 mg/kg	-	360	3.9	-
21.4 1955	85	B	0	2 x 20 mg/kg	-	325	0.8	-
				mean ± S.E.:				
12.5 1955	86	B	4	2 x 20 mg/kg	375	363	0.8	37.5
12.5 1955	87	B	4	2 x 20 mg/kg	398	360	2.1	-
16.5 1955	88	B	4	2 x 25 mg/kg	395	365	0.9	38.5
16.5 1955	89	B	4	2 x 25 mg/kg	360	337	0.8	39
				mean ± S.E.:				

TABLE 32 Contd:

gesterone 18 hr and 1 hr before the blood collection. Blood collected for 15 min. under pentobarbitone anaesthesia.

l.adrenal mg/kg b. wt.	Corticosterone secretion µg/g adr./min.	Corticosterone secretion µg/hr/adr./kg b.wt.	% recovery	NaOH fluorescence
61	22.2	81	68	x:?
56	24.0	80	73	x:++
65	22.4	83	75	x:-
60 ± 3.1	22.9 ± 0.61	81 ± 1		
81	7.9	39	60	x:?
71	8.1	34	64	x:?
95	7.7	44	58	x:-
91	8.3	45	62	x:-
85 ± 5.8	8 ± 0.15	41 ± 10		

described by Gelin (1956) in experiments on dextranes of high molecular weight. It is likely that in these experiments cholesterol did either not penetrate to the site in the tissue where it could be used for corticosterone synthesis, or if it penetrated, that the blood supply to the adrenal or the pituitary was lowered and normal response of these glands inhibited. Therefore, none of these experiments are contradicting the theory that it is cholesterol to which the beneficial effect of normal rat plasma is due.

disprove

g. Progesterone: Hechter et al. (1951) have considered progesterone to be an intermediate in the synthesis of corticosterone. It was therefore tried to infuse progesterone solutions into rats pretreated with hexoestrol. Results of such experiments are given in Table 30. When bovine albumin or plasma from hexoestrol treated rats was used as vehicle and the solutions were infused intravenously, no change in adrenocortical secretion was observed. When infused into the aorta, it caused a marked inhibition of corticosterone secretion. The infusion did not cause rise in blood pressure, and they caused oedema in the region of the psoas muscles. When normal rat plas-

TABLE 33

Cholesterol feeding

All rats were fed for 14-17 days on a diet containing 1.36% cholesterol and 8.6% olive oil. Corticosterone secreted by the left adrenal of

Blood coll. Date	Rat No.	No. of Hex. inj.	Cholesterol oil diet, no. of days	b. wt. (g) at start of Spec. diet	b. wt. (g) at start of Hex. inj.	b. wt. (g) at end	mean body temp. (°C)	plasma (g)	Cholesterol in arterial blood (mg %)	l. adrenal mg/kg b. wt.
15.10 1956	329	0	14	330	-	353	37	1.4	67	58
15.10 1956	330	0	14	324	-	359	38.8	0.9	-	61
						mean:				59.5
15.10 1956	326	5	14	350	356	332	37	2.4	7.5	101
15.10 1956	327	5	14	331	324	306	39	1.4	8.5	96
15.10 1956	328	5	14	326	304	283	34.5	1.8	-	92
18.10 1956	332	5	17	327	365	340	39.5	1.4	7	87
18.10 1956	333	5	17	364	365	332	37.5	2.2	9.3	63
18.10 1956	334	5	17	356	370	332	37.1	3.0	4.3	105
								mean ± S.E.:		90.6 ± 6.8

TABLE 33 Contd:

control rats and of hexoestrol treated rats (0.4 mg/kg/day subcutaneously on 5 consecutive days immediately prior to the blood collection. Blood collected under pentobarbitone anaesthesia for 15 min.

Rat No.	Corticosterone secretion µg/g adr./min	Corticosterone secretion µg/hr/adr./kg b.wt.	% re-covery	NaOH-fluor-escence	Histology
329	29.1	101	76	x:+	all three layers lipid laden
330	36.3	133	71	x:+	all three layers lipid laden
	32.7	117			
326	21.1	128	-	x:+	lipid depletion in fasciculata
327	15.4	89	70	x:+	some lipid depletion in fasciculata
328	9.8	54	65	x:-	some lipid depletion in fasciculata
332	14.4	75	75	x:-	some lipid depletion in fasciculata
333	19.2	72	76	x:+	some lipid depletion in fasciculata
334	10.5	66	76	x:+	lipid depletion in fasciculata
	15.1 ± 1.8	80.6 ± 11.9			

ma was used as vehicle for intravenous administration of progesterone, the secretory capacity increased to a similar extent as in the experiments with normal rat plasma only. When normal rats were given an intravenous infusion of progesterone the secretion rate of their adrenals was at the lower limit of normal.

2. Long lasting pretreatment.

a. Progesterone: One group of experiments on hexoestrol depressed adrenals was carried out with progesterone administered subcutaneously 18 and one hour before the blood collection. No restoration of the inhibited secretion was obtained. When normal rats received the same pretreatment with progesterone, their adrenal secretion lay on the lower limit of normal.

b. Cholesterol feeding. An oil rich diet containing 1.4% cholesterol was fed for 14-17 days. Control rats fed in this way had a secretion rate of 32.7 µg corticosterone/g adrenal/min. (Table 33). Their livers showed fatty infiltration and all three layers of the adrenal cortex were laden with lipids. The blood cholesterol was normal and not elevated. Rats kept for 14-17 days on the same diet, but given daily injections of hexoestrol (0.4 mg/kg) on the five days preceding blood collection, had a corticosterone secretion of only 12.5 µg/g adrenal/min. The adrenals of these rats showed lipid loss and their blood cholesterol was only 7.3 ± 1 mg/100 ml. Under these circumstances, it was not surprising that the corticosterone secretion was not increased after feeding of cholesterol.

c. Erucic acid. When rats are fed for four weeks on a diet containing a high proportion of

TABLE 33a

Erucic acid

Corticosterone secreted by the left adrenal of rats fed for 14 to 16 days with a diet containing 10% erucic acid. Blood collected

Blood coll. Date	Rat No.	Strain	Duration of treatment (days)	b. wt. at start	b. wt. at end	plasma (g)	mean body temp. (°C)	l. adrenal mg/kg b. wt.
Controls under pentobarbitone (Group 3) see Table 5						mean ± S.E.:		67.7 ± 3.1
21.6 1956	250	E	14	216	230	1.3	36.5	70
21.6 1956	252	E	14	242	250	1.8	38	71
21.6 1956	253	E	14	250	258	1.8	35	82
23.6 1956	256	E	16	230	240	1.7	?	75
16.7 1956	271	T	18	223	234	1.5	37	97
16.7 1956	272	T	18	247	276	0.8	41	89
mean ± S.E.:								81 ± 4
Significance of difference from controls:								$P < 0.05$

Table 33a

in pentobarbitone anaesthesia for 15 minutes.

Rat No.	Corticosterone secretion		% recovery	NaOH-fluorescence	Histology
	µg/g adr./min.	µg/hr/adr./kg b.wt.			
	33.1 ± 1.7	135 ± 10.3		(n = 13)	
250	16.2	68	78	x:+	
252	34.8	149	73	x:++	All three layers extremely lipid laden.
253	25.7	126	73	x:+	
256	44.6	200	89	x:+	
271	38.0	222	66	x:?	
272	21.8	115	71	x:-	
	30.0 ± 4.6	147 ± 25			
	P > 0.1	P > 0.1			

TABLE 33b

Corticosterone secreted by the left adrenal of rats (B-strain) fed for 21 or 28 days with a diet containing 12% erucic acid and injected with hexoestrol (0.4 mg/kg/day) on the five days preceding blood collect-

Date of blood coll.	Rat No.	No. of Hex. inj.	Erucic acid diet: No. of days	b. wt. (g) at start of: spec. diet	b. wt. (g) at start of: Hex. inj.	b. wt. (g) at end	Mean body temp. (°C)	Plasma (g)	Cholesterol in arterial blood (mg %)	l. adrenal mg/kg b.wt.
n = 40	Hexoestrol controls:						mean ± S.E.:			93 ± 4
4.3 1957	363	5	21	271	278	245	37	2	10	110
4.3 1957	364	5	21	241	230	198	37	2	5	114
4.3 1957	365	5	21	288	265	232	38	2.2	5	158
4.3 1957	366	5	21	255	254	225	38	1.7	7	112
11.3 1957	367	5	28	280	325	293	37	1.7	18	103
11.3 1957	368	5	28	256	304	255	38	1.3	9	105
11.3 1957	369	5	28	238	270	240	38	1.5	4	98
11.3 1957	370	5	28	272	315	275	36	1.8	4	141
							mean ± S.E.:			117 ± 7.4
Significance of difference from Hexoestrol controls:										P < 0.01

TABLE 33b

ion. (Pentobarbitone anaesthesia, collection time 15 minutes.)

Rat No.	Corticosterone secretion		% recovery	NaOH-fluorescence	Histology
	µg/g adr./min.	µg/hr/adr./kg b.wt.			
	13.7 ± 0.88	74.3 ± 6.5			
363	22.0	145	70	x:+	All adrenals contain sudanophilic lipids in the fasciculata though the distribution is uneven and patchy.
364	21.5	148	55	x:+	
365	14.0	133	65	x:?	
366	16.9	114	62	x:?	
367	12.5	78	54	x:++	
368	13.5	85	71	x:+	
369	20.6	122	60	x:+	
370	12.7	108	68	x:?	
	16.7 ± 1.18	120 ± 8.7			
	P > 0.1	P < 0.01			

rape seed their adrenals are much whiter than those of normal rats, the cortex becomes hypertrophied and its lipid content increases (Kennedy and Purves, 1941; Carroll, 1949). A diet containing 25% of rape seed oil increased adrenal cholesterol by about 300% (Carroll, 1951). The factor in rape oil which affects adrenal cholesterol in the rat is erucic acid (Carroll, 1953).

Since it had been observed that rape oil can antagonise the depressing effect of stilboestrol on adrenal cholesterol (Carroll and Noble, 1952), an experiment was devised in which rats were fed for several weeks with erucic acid and injected simultaneously with hexoestrol on the last 5 days of the experiment. The corticosterone concentration of adrenals of rats fed with erucic acid only was 30.0 ± 4.6 µg/g adr./min. and 147 ± 25 µg/hr/adr./kg b. wt. (Table 33a). The figures lie within the normal range. All three zones of the adrenal cortices were laden with lipids. When hexoestrol injections were given on the 5 days preceding blood collection the corticosterone secretion amounted only to 16.7 ± 1.2 µg/g adr./min. It was about 30% higher than the mean secretion of 40 rats treated with hexoestrol only. The secretion rate per hour per adrenal per kg b. wt. was calcu-

lated as 120 ± 8.7 and was about 70% higher than that of the hexoestrol controls. This difference is statistically significant. The blood cholesterol concentration of all rats treated with the combination of erucic acid and hexoestrol was depleted by more than 90%. The adrenal cholesterol was estimated in the last 4 rats of Table 33b. It was found to lie between 0.6% and 0.8%, that means it was also depleted by 80-90%. Thus erucic acid was not able to antagonise the inhibitory effect of hexoestrol on adrenal cholesterol storage and this explains why corticosterone secretion was depressed. The histological picture of adrenals of rats treated with a combination of erucic acid and hexoestrol showed lipid depleted patches in the zona fasciculata. The amount of sudanophilic lipids present in the glands was however, much higher than in rats treated with hexoestrol alone.

Discussion

The foregoing observations support the theory that it is by interference with cholesterol synthesis that hexoestrol exerts its inhibiting effect on the rat adrenal cortex. The fact that infusion of normal rat's plasma restored normal corticosterone secretion within a few minutes indicates the integrity of the enzyme systems synthesizing corticosterone. Since plasma from hex-

oestrol treated rats is inactive and known to contain only small amounts of cholesterol, the missing factor supplied by normal plasma is in all probability cholesterol.

The amounts of ACTH present in normal rat plasma obtained in ether anaesthesia (less than 4 m.u./100 ml. Sydnor and Sayers, 1954) cannot account for improved adrenocortical function. Vogt (1955) observed that infusion of 5 or 10 m.u. ACTH into hexoestrol pretreated rats immediately before collection of adrenal vein blood, did not significantly increase corticosterone output.

The fact that suspensions of lipoproteins or cholesterol did not restore normal secretion in a hexoestrol inhibited adrenal is no proof that lack of cholesterol is not the cause of the depression. All solutions exerted toxic effects on circulation or caused signs of brain damage, probably due to unsuitable particle size.

Feeding of cholesterol did not increase the plasma cholesterol concentration in normal rats and did not prevent the fall of plasma cholesterol in hexoestrol treated rats. Therefore, it was not surprising to find that the secretory capacity of the adrenal cortex was unaffected. The same was the case in the experiments where erucic acid

was fed.

Progesterone has been shown to be utilized by the perfused cow adrenal in the synthesis of cortical hormones. In the present experiments, intravenous infusions did not influence and intra-arterial infusions inhibited cortical secretion. It is difficult to interpret these results. Since progesterone is known to disappear very rapidly from the circulation (Zarrow et al. 1954) the material infused intravenously might have been trapped before it reached the adrenals.

On the other hand, after infusion into the aorta fluid and presumably progesterone, accumulated in the musculature round the aorta and in periadrenal tissue. It is therefore likely that some progesterone also entered adrenal tissue. The effect of this infusion was a depression of adrenal function. One might speculate that this inhibition was due to a biochemical mechanism. Brownie, Grant and Davidson (1954) demonstrated that progesterone inhibits 11-hydroxylation of desoxycorticosterone to corticosterone in the mitochondria of ox adrenal cortex. It is however, impossible to exclude the alternative that capillary circulation in the adrenal was impaired by

particles of progesterone, carried in the infusion fluid.

Another measure to antagonise lipid loss and functional depression caused by hexoestrol is administration of amphenone 'B' (Vogt, 1957). This substance (1,2-bis (p-amino-phenyl)-2 methyl-propanone-1, dihydrochloride), when fed to rats, causes a very pronounced hypertrophy of the adrenals and an increase of adrenal cholesterol by several hundred per cent (Hertz et al. 1955). Such enlarged adrenals have a normal secretory capacity per g tissue, and secretion per kg b. wt. is much increased. Rats were given 4 daily injections of hexoestrol by which time stainable lipids have disappeared from the fasciculata. The injections were then combined with a daily dose of 0.2 g/kg amphenone by stomach tube. After that treatment the mean corticosterone secretion of the 4 rats investigated was 24.3 $\pm$ 1.6 µg/g adrenal and 142 $\pm$ 5 µg/hr/adr./kg b. wt. That means that secretion per g adrenal was back to normal. The secretion per kg b. wt. was even larger than that of normal control rats. In spite of improved adrenal function these rats were however, jaundiced and altogether in poor health.

see p 39

Summary

1. When corticosterone secretion of the rat adrenal was inhibited by hexoestrol, intravenous infusion of plasma from normal rats restored secretion to normal within a few minutes.

2. No such effect was observed when the plasma of normal rats was replaced by plasma from rats which had themselves been injected with hexoestrol, or by solutions of bovine albumin.

3. This suggested that the responsible factor for the beneficial effect of normal rats plasma is its cholesterol content.

4. In rats injected with hexoestrol, feeding of cholesterol or erucic acid neither restored the cholesterol content of the blood nor the corticosterone secretion from the adrenals.

5. Progesterone given intravenously did not improve the secretion of hexoestrol inhibited adrenals and given arterially it caused a further decrease in the secretion rate.

6 Amphenone

CHAPTER 9

The Concentration of Corticosterone in Rat Adrenals under different Experimental Conditions

The determination of the quantities of hormones present in an endocrine gland is a widely used method for the study of the activity of the gland. The application of this method to the adrenal cortex was until recently, limited by the very small amounts of biologically active corticosteroids present in it at any time. A second general difficulty of this method is the fact that the relationship between hormone content and secretory activity was quite unknown.

The first limitation, set by the low corticosteroid concentration in adrenal tissue, has been overcome by the micro methods developed in steroid chemistry during the last years, especially the paperchromatographic techniques. These methods make it possible to work with 80 to 200 mg of tissue and under very favourable conditions a single rat adrenal is sufficient.

The second difficulty, namely the interpretation of the results in terms of secretory activity can partly be overcome by measuring the amount of hormone released into adrenal vein blood under comparable experimental conditions.

The plan of the experiments was to measure the corticosterone concentration in rat adrenals under different experimental conditions, and to compare the figures obtained with the amount of corticosterone secreted in a given period of time under similar conditions.

The adrenals were obtained either from rats which were killed by rapid decapitation, "unstressed rats", or from animals which were killed by exsanguination under ether anaesthesia, "stressed rats". Furthermore, adrenal corticosterone was estimated after pretreating rats with drugs or hormones which produce structural and functional changes in the glands. The substances used were adrenocorticotrophic hormone (ACTH), hexoestrol, amphenone "B", adrenaline and histamine.

Methods

Eleven groups of experiments were carried out. For each single estimation 2 to 20 adrenals were pooled and the corticosteroids extracted, purified and estimated as described under "Methods"

1. Untreated Controls

The first 4 groups of experiments were performed on animals not pretreated with any drug. The rats of groups 1 and 2 belonged to the B-, Bi- and

Es-strain. All rats were kept in individual cages and undisturbed for 24 hours; one group (see Table 34, "unstressed rats", untreated controls) was killed by rapid decapitation, the others (see Table 35, "stressed rats", untreated controls) by bleeding in ether anaesthesia from a cannula in the carotid artery, into which heparin (1000 i.u./kg) had been injected. Four to six adrenals were pooled for each estimation, corresponding to 68 to 125 mg of tissue. Another experiment on untreated controls was done on "stressed" and "unstressed" rats of the Ew-strain (see Table 36).

2. Pretreatment of long duration

a. Adrenocorticotrophic hormone (ACTH)

In order to study the corticosterone content of adrenals enlarged after pretreatment with ACTH, 16 rats (B-strain) received ten daily injections of long acting ACTH'Z' (1 i.u./rat/day, s.c.). The animals were killed 24 hours after the last ACTH injection, eight of them by rapid decapitation, eight of them by bleeding in ether anaesthesia. The corticosterone concentration was estimated in 8 groups, each consisting of four pooled adrenals (116-128 mg tissue) (see Table 37).

b. Hexoestrol

The experiments with hexoestrol were done on thirteen rats (Es-strain) pretreated with five

daily s.c. injections of 0.2 ml of a 0.2% solution in arachis oil/kg. All animals were killed by exsanguination under ether 24 hours after the last injection. The corticosterone concentration was estimated in 5 groups, consisting of four or six adrenals each (see Table 38).

c. Amphenone "B"

To investigate the effect of amphenone "B" (further on called 'amphenone') on adrenal corticosterone stores, ten rats (B-strain) were fed for ten days with 0.4 ml of a 5% watery solution/100 g body weight/day. The animals were killed 24 hours after the last drug administration, six of them by rapid decapitation, four of them by bleeding under ether. The corticosterone content was estimated in groups made up of four pooled adrenals ("unstressed rats") or of three or two pooled adrenals ("stressed rats"), corresponding to between 54 and 110 mg of tissue (see Table 39). Finally, an experiment was carried out on twenty-two "unstressed rats", in which approximately 400 mg of adrenal tissue were used for each single estimation. The rats were kept in individual cages in a thermostatically (25°C) controlled room for a period of eleven days. Six of them were pretreated for ten days with ACTH, six of them with amphenone, as described above. The

remaining ten served as untreated controls. All rats were killed by rapid decapitation on the eleventh day and the adrenals of the rats of each group pooled for the corticosterone estimations (see Table 40).

3. Pretreatment of short duration

An experiment was conducted in order to study the effects of single injections of ACTH, adrenaline or histamine on the corticoid stores of the adrenals. Whereas in all previous experiments the glands had been examined 24 hours after the last drug administration, in the first of these experiments they were obtained after an interval of two or three hours. Seventeen rats of the B-strain were used. They were kept for four days in individual cages in a thermostatically controlled room (at 25°C) and received three daily subcutaneous injections of 0.2 ml of a 0.9% solution of NaCl. On the morning of the fourth day two rats (group a) served as controls and were injected with 0.2 ml of saline subcutaneously 2 hours prior to decapitation. The five rats of group b received 1 i.u. of ACTH'Z' subcutaneously 3 hours before death. The five rats of group c were each injected subcutaneously with 200 µg l-adrenaline base/kg body weight 2 hours before death and the five rats of group d with 20 mg of histamine acid phosphate/kg

body weight given subcutaneously 2 hours before death. All animals were killed by rapid decapitation. Treatment of groups e and f will be described under 'results'.

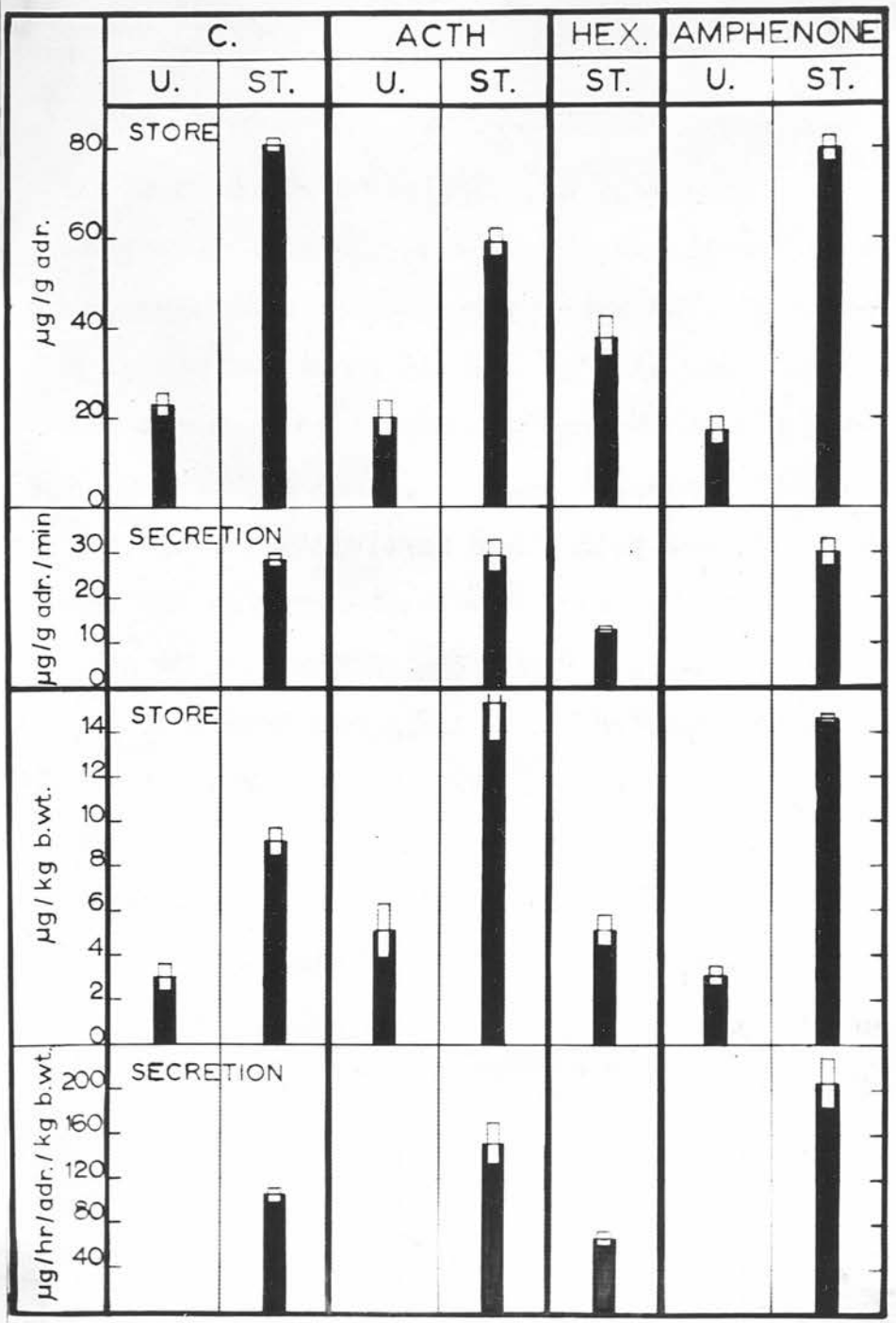
4. Comparison between hormone store and secretion rate

Information on the secretory performance of adrenals after standard pretreatment was obtained by measuring the amounts of corticosterone secreted into adrenal vein blood in a given time (see Chapters 4, 5 and 7). The stress of laparotomy under urethane or pentobarbitone anaesthesia was considered to be comparable to exsanguination from the carotid artery in ether anaesthesia. Under these circumstances the secretion approaches the maximal capacity of the adrenal (see Chapter 5, p. 73).

In some cases, the corticosterone content of glands was estimated after a sample of adrenal vein blood had been collected. Figures obtained in this manner had, however, a wide range and therefore were not used for evaluation.

Attempts at obtaining adrenal vein blood from rats under "resting conditions" have so far failed. The introduction of a permanent cannula, which connects the left renal vein with a lumbar vein, passing twice through the flank of the rat, is techni-

Figure XIV



Comparison between the amount of corticosterone stored and secreted by rat adrenals.

C = Controls; U = "Unstressed rats"; St. = "Stressed rats".

cally possible. Clotting however, occurs within a few hours, despite repeated injections of heparin into the cannula.

Results

The main C_{21}-corticosteroid present in rat adrenals has the same R_F-value as corticosterone. The same holds for the main steroid of adrenal rats blood. In addition, two minor components were observed in the glands; these also occur in adrenal vein blood. One of them is only somewhat more polar than corticosterone and was eluted together with corticosterone for the quantitative assays. The other one is in the region of Bush's compound X. Sometimes traces of a fluorescing spot were visible in the 11-dehydrocorticosterone region of the chromatograms which had been sprayed with NaOH. Under the conditions of these experiments no other steroid appeared in the chromatograms. The ultra violet absorbing spots of none steroidal nature observed in adrenal vein blood and arterial blood extracts of amphenone pretreated rats (Vogt, 1957) did not occur in the extracts of the glands of these rats (see also Chapter 3).

The results of the quantitative estimations are listed in the Tables 34, 35, 36, 37, 38, 39, 40 and 41 and summarized in Fig. XIV and XV.

TABLE 34

Corticosterone stores in adrenals of rats kept undisturbed for 24 hours and then killed by rapid decapitation: "unstressed rats", untreated controls.

Date	Expt. No.	Strain	mean b.wt. rat (g)	No. of adr. glds.	Adr. tissue (mg)	mg adr./ kg b. wt.	CORTICOSTERONE STORE		% re-cov-ery	NaOH-fluor-escence
							µg/g adr.	µg/kg b. wt.		
14.2 1956	XLII	Bi	407	6	119	98	25.2	2.48	74	x:+?
14.2 1956	XLIII	B	318	6	118	124	22.9	2.83	74	x:+?
20.2 1956	XLV	B	302	6	114	126	24.1	3.04	75	x:+?
23.2 1956	XLVII	B	227	5	98	189	28.6	4.95	69	x:-
4.12 1956	LXV	B	344	4	84	122	14.4	1.75	79	x:+?
			mean ± S.E.:			132±17.5	23.0±2.73	3.01±0.62		

TABLE 35

Corticosterone stores in adrenals of rats killed by bleeding in ether anaesthesia from the cannulated carotid artery: "stressed rats", untreated controls.

Date	Expt. No.	Strain	mean b.wt. rat (g)	No. of adr. glds.	Adr. tissue (mg)	mg adr./ kg b. wt.	CORTICOSTERONE STORE µg/g adr.	CORTICOSTERONE STORE µg/kg b. wt.	% recovery	NaOH-fluorescence
"unstressed rats" Untreated controls (n = 5)			mean ± S.E.:			132±17.5	23.0±2.73	3.01±0.62		
1.6 1955	I	Bi	365	6	125	115	85.6	9.82	82	not done
7.6 1955	II	Bi	295	4	75	127	80.0	10.20	76	not done
13.6 1955	VI	Bi	342	4	72	105	83.3	8.75	72	x:++
13.6 1955	VII	Bi	410	4	68	83	81.0	6.70	78	x:++
7.2 1956	XXXVIII	Bi	400	6	123	103	87.0	8.34	82	x:++ A:?
22.6 1955	XIV	Es	333	4	72	108	76.5	8.30	-	x:++
23.2 1956	XLVI	B	249	6	110	148	80.4	11.85	77	x:++
7.2 1956	XXXIX	B	351	6	108	103	75.8	7.78	74	x:++ A:?
20.2 1956	XLIV	B	275	6	98	119	80.6	9.58	75	x:++
			mean ± S.E.:			112±7.3	81.1±1.26	9.04±0.58		
Significance of difference from controls:						P > 0.1	P < 0.01	P < 0.01		

TABLE 36

Corticosterone stores in adrenals of "stressed" and "unstressed" rats of the Ew-strain.

Date	Expt. No.	Treatment	Mean b.wt. rat (g)	No. of adr.	Adr. tissue (mg)	µg adr/kg b.wt.	CORTICOSTERONE STORE µg/g adr.	CORTICOSTERONE STORE µg/kg b.wt.	% recovery	NaOH fluorescence
20.12 1955	XXXI	Untreated controls "stressed rats"	317	6	97	104	67.0	6.85	68	x:-
1.2 1956	XXXVI		311	6	93	100	48.4	4.84	63	x:-
9.2 1956	XL		305	6	87	94	65.5	6.23	77	x:++
		Mean ± S.E.:				99 ± 3.4	60.3 ± 6.4	5.97±0.69		
30.1 1956	XXXIII	Untreated controls "unstressed rats"	330	6	97	98	13.4	1.31	50	x:-
30.1 1956	XXXIV		356	6	109	102	12.9	1.31	63	x:-
9.2 1956	XLI		296	8	124	114	24.2	2.76	93	x:?
		Mean ± S.E.:				105 ± 5.5	16.8 ± 3.9	1.79 ± 0.5		
Significance of difference:						P > 0.1	P < 0.01	P < 0.01		

1. Untreated controls

The corticosterone content of adrenals of unstressed rats was estimated as 23.0 ± 2.73 µg/g adrenal and 3.01 ± 0.62 µg/kg body weight. When rats of the same strains were killed by exsanguination in ether anaesthesia the corticosterone content of their adrenals was much higher, namely 81.1. ± 1.26 µg/g adrenal and 9.04 ± 0.58 µg/kg body weight. Whereas adrenal extracts from "unstressed rats" did not contain visible amounts of compound X, every extract of adrenals from "stressed rats" showed an intense fluorescence in the X-region of the chromatograms, and two of them also in the 11-dehydrocorticosterone region. There was remarkably little variation between individual estimates, although the rats originated from three different sources and the experiments were done during a period of 1½ years.

There was however, one group of rats (Ew-strain), in which the figures were consistently about 30% lower, under resting conditions as well as after the brief period of stress (see Table 36). In these animals too, lethal haemorrhage led to a 4-fold increase of the corticosterone stores.

The mean corticosterone secretion of forty-three normal rats was determined as 28.3 µg/g ad-

TABLE 37

The effect of 10 daily s.c. injections of long acting ACTH-Z (1 i.u./rat/day) on the corticosterone stores in adrenals of "unstressed" and "stressed" rats. All animals were killed 24 hours after the last drug administration.

Date	Expt. No.	Strain	Treatment	Mean b.wt. rat (g)	No. of adr. glds.	Adr. tissue (mg)	mg adr./ kg b. wt.	CORTICOSTERONE STORE		% re-covery	NaOH fluor-escence
								µg/g adr.	µg/kg b.wt.		
(n = 5)			Untreated controls "unstressed rats	mean ± S.E.:			132±17.5	23.0±2.73	3.01±0.62		
1.3 1956	XLVIII	B	ACTH treated "unstressed rats"	308	4	126	205	14.3	2.92	82	x:-
1.3 1956	IL	B		267	4	128	236	12.9	3.09	-	x:-
5.3 1956	LIV	B		228	4	125	275	23.2	6.37	77	x:-
5.3 1956	LV	B		241	4	126	262	30.1	7.90	81	x:+
			mean ± S.E.:				245±17	20.1±4.2	5.07±1.2		
Significance of difference from controls:							P < 0.01	P > 0.1	P > 0.1		
(n = 9)			Untreated controls "stressed rats"	mean ± S.E.:			112±7.3	81.1±1.26	9.04±0.58		
1.3 1956	L	B	ACTH treated "stressed rats"	278	4	127	229	64.2	14.65	90	x:+
1.3 1956	LI	B		263	4	118	224	55.9	12.50	85	x:-
5.3 1956	LII	B		202	4	120	298	65.8	19.60	-	x:-
5.3 1956	LIII	B		211	4	116	276	52.2	14.40	73	x:-
			mean ± S.E.:				257±18	59.5±3.3	15.29±1.7		
Significance of difference from controls:							P < 0.01	P < 0.01	P < 0.01		

renal/minute. The store of 81.1 µg corticosterone per g adrenal would thus represent the amount secreted in 2.9 minutes.

There was no obvious difference between the amounts of sudanophilic lipids present in adrenals of "stressed" or "unstressed" rats. This is not surprising considering that the total procedure lasted approximately 15 minutes.

2. Pretreatment of long duration

a. Adrenocorticotrophic hormone

In this experiment, 10 daily subcutaneous injections of 1 i.u. ACTH-Z/rat caused an increase in adrenal weight of about 100%. The cells of the enlarged, well vascularised adrenal cortex were rich in sudanophilic lipids. The corticosterone content of such adrenals was estimated under resting conditions as 20.1 ± 4.2 µg per g adrenal, and 5.07 ± 1.2 µg per kg body weight. This value does not differ from that of normal adrenals under the same conditions. After a brief period of stress there was a rise to 59.5 ± 3.3 µg per g adrenal and 15.3 ± 1.7 µg per kg body weight. Thus an adrenal, highly enlarged by chronic administration of ACTH contains significantly less corticosterone per g tissue, than does a normal adrenal after the

same stress, but the amounts of corticosterone stored per kg body weight are significantly increased.

The secretory performance of adrenals of another group of rats, which were given 11 to 14 daily preoperative injections of 1 i.u. ACTH-Z was estimated as 29.1 µg corticosterone per g adrenal per minute (see Table 10). Thus the amount of corticosterone present in adrenals which were chronically stimulated by ACTH would be adequate to cover only a 2 minutes secretion. The validity of this figure is however, doubtful because the two groups were not strictly comparable. The rats used for the gland experiments had a mean body weight of 261 g and a mean adrenal weight of 250 mg/kg body weight, those used for blood collection a mean body weight of 320 g and a mean adrenal weight of 85 mg/kg body weight. It is questionable whether the secretory capacity per g tissue of the extraordinarily enlarged adrenals of the smaller rats would have been as high as that of the less hypertrophied adrenals of the larger rats.

b. Hexoestrol

The structural and functional changes caused by hexoestrol in rat adrenals are described in Chapter 7. Table 38 shows the results of experiments

TABLE 38

The effect of 5 daily s.c. injections of hexoestrol (0.4 mg/kg/day, as 0.2% solution in arachis oil) on the corticosterone stores in adrenals of "stressed rats". All animals were killed 24 hr after the last drug administration.

Date	Expt. No.	Strain	Treatment	Mean b.wt. rat (g)	No. of adr. glds.	Adr. tissue (mg)	mg adr./ kg b. wt.	CORTICOSTERONE STORE		% re-covery	NaOH-fluor-escence
								µg/g adr.	µg/kg b.wt.		
(n = 9)			Untreated controls "stressed rats"	mean ± S.E.:			112±7.3	81.1±1.26	9.04±0.58		
20.6 1955	IX	Es	Hexoestrol treated "stressed rats"	369	4	105	142	45.0	6.37	60	x:+
20.6 1955	X	Es		363	4	89	123	30.4	3.72	58	x:+
22.6 1955	XII	Es		356	6	129	122	30.2	3.68	64	x:+
27.6 1955	XV	Es		298	6	122	136	53.0	7.20	-	x:-
30.6 1955	XVI	Es		311	6	132	141	32.5	4.60	69	x:?
mean ± S.E.:							133±3.9	38.2±4.4	5.11±0.68		
Significance of difference from controls:							P < 0.05	P < 0.01	P < 0.01		

on the corticosterone stores in such glands. All glands were obtained after bleeding the rats in ether anaesthesia. The mean corticosterone content per g adrenal was 38.2 ± 4.4 µg;per kg body weight 5.11 ± 0.68 µg. Thus in an adrenal, which is depleted of its lipids and functionally inhibited by hexoestrol, the corticosterone stores are also reduced by about a half.

The mean secretion of these glands was 12.7 µg per g adrenal per minute. Therefore, like normal adrenals, hexoestrol-inhibited adrenals store an amount of corticosterone, equivalent to that secreted in 3 minutes.

c. Amphenone "B"

Feeding amphenone (1,2-bis-(p-aminophenyl)-2-methylpropanone-1) to rats for ten to seventeen days leads to a very pronounced hypertrophy of the adrenals, accompanied by an increase in lipid content, and especially cholesterol content of the glands (Hertz et al. 1955). These changes are absent in hypophysectomised, ACTH maintained rats (Hertz and Tullner). The secretory capacity of such adrenals, when expressed as µg per g gland, was found to be at the upper limit of normal. When expressed as µg per kg body weight there was an increase of about 150% over the normal value (Vogt,

TABLE 39

The effect of 10 daily p.o. doses of amphenone "B" (0.4 ml of a 5% watery solution/100 g b. wt./day) on the corticosterone stores in adrenals of "unstressed" and "stressed" rats. All animals were killed 24 hours after the last drug administration.

Date	Expt. No.	Strain	Treatment	Mean b.wt. rat (g)	No. of adr. glds.	Adr. tissue (mg)	mg adr./ kg b. wt.	CORTICOSTERONE STORE		% recovery	NaOH fluorescence
								µg/g adr.	µg/kg b.wt.		
(n = 5)			Untreated controls "unstressed rats"	mean ± S.E.:			132±17.5	23.0±2.73	3.01±0.62		
30.4 1956	LIX	B	amphenone treated "unstressed" rats	254	4	96	190	16.7	3.16	> 60	x:-
30.4 1956	LX	B		285	4	110	193	12.7	2.46	> 62	x:-
30.4 1956	LXI	B		312	4	108	171	21.3	3.70	72	x:-
mean ± S.E.:							185±7.5	16.9±2.9	3.11±0.42		
Significance of difference from controls:							P < 0.05	P > 0.1	P > 0.1		
(n = 9)			Untreated controls "stressed rats"	mean ± S.E.:			112±7.3	81.1±1.26	9.04±0.58		
7.4 1956	LVI	B	amphenone treated "stressed rats"	275	3	71	177	83.8	14.8	68	x:-
7.4 1956	LVII	B		275	3	84	190	73.8	14.6	68	x:-
7.4 1956	LVIII	B		298	2	54	181	79.7	14.4	64	x:-
mean ± S.E.:							183±4.4	79.1±3.4	14.6±0.14		
Significance of difference from controls:							P < 0.01	P > 0.1	P < 0.01		

1957). Release of ACTH has been suggested as possible mechanism of action (Hertz et al. 1955). As it is however, not possible to imitate the whole effect of emphenone by ACTH injections, a direct action of amphenone on the metabolism of the adrenal cortex was postulated by Vogt (1957). The fact that amphenone is able to restore normal secretion in an hexoestrol inhibited adrenal (see Chapter 7) is further evidence in favour of this theory.

Adrenals of amphenone fed rats, obtained after killing the animals by quick decapitation, contained 16.9 ± 2.9 µg corticosterone per g tissue, and 3.11 ± 0.42 µg per kg body weight. In stressed rats, 79.1 ± 3.4 µg per g adrenal, and 14.6 ± 0.14 µg per kg body weight were present (see Table 39). Only the last figure is significantly higher than that of normal rats.

The mean secretion of rats, fed for ten days with amphenone was 29.9 µg per g adrenal per minute. The amount stored would therefore be secreted in 2.6 minutes.

The amounts of corticosterone present in 4-6 pooled adrenals of "unstressed rats" was usually less than 2 µg, and the ultra violet absorption of the spots in the chromatograms usually very feeble. With samples, containing steroids in this order of

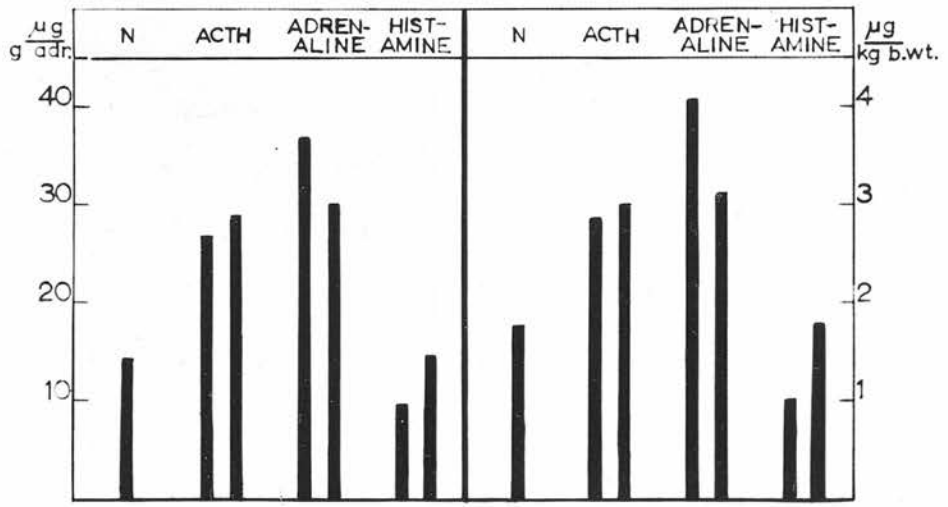
TABLE 40

Effect of ten daily doses of ACTH-Z or amphenone "B" on the corticosterone stores in adrenals of "unstressed rats" from the same strain. A large number of adrenals were pooled for each group. All animals were killed 24-hours after the last drug administration.

Expt. No.	Strain	Treatment	Mean b.wt. rat (g)	No. of adr.	Adr. tissue (mg)	µg adr/kg b.wt.	CORTICOSTERONE STORE		% recovery	NaOH fluorescence
							µg/g adr.	µg/kg b.wt.		
LXIII	0	Untreated controls	302	20	393	130	7.4	0.96	87	x:+
LXII	0	1 i.u. long acting ACTH/ rat/day s.c. for 10 days	276	12	373	225	11.5	2.60	87	x:+ A:?
LXIV	0	20 mg of amphenone B/100 g. b.wt./day, fed for 10 days	284	12	402.5	241	12.2	2.93	83	x:+ A:?

magnitude, impurities in the corticosterone region of the chromatograms could lead to over-estimates of as much as 50% (see p.37). Therefore an experiment was carried out, in order: (1) to obtain reliable identification of corticosterone in glands of "unstressed rats", (2) to exclude errors in quantitative estimations, and (3) to establish whether under these conditions there is a marked difference between the corticosterone concentration in adrenals of control rats and those of rats pretreated for ten days with ACTH or amphenone. To obtain a sufficient amount of steroids, 12 to 20 adrenals were pooled for each determination. The results are shown in Table 40). All three extracts contained enough corticosterone to be clearly visible by ultra violet absorption. The glands of the ACTH or amphenone pretreated rats contained somewhat more hormone per unit of weight than those of the untreated rats. The difference in the stores per kg body weight was more pronounced. The figures were generally lower than in previous experiments, particularly those for the rats which had no pretreatment. Since the rats of this experiment belonged to the O-strain which had not been used before, it is difficult to decide whether the lower figures indicate an over-estimate in the experiments carried out on less tissue or have to be attributed

Figure XV



The effect of a single injection of ACTH, adrenaline or histamine on the amount of corticosterone found in rat adrenals. (N = normal controls).

to differences between strains.

3. Pretreatment of short duration

In the experiments described so far, the last injection of any drug was given 24 hours before extraction of the glands. They were therefore only suitable for the detection of changes which had developed slowly in the adrenals. The large difference between the amounts of corticosterone stored in a resting adrenal and in an adrenal obtained after a 10-15 minutes severe stress suggests that ACTH release leads very quickly to an increase of the corticosterone stores. It seemed of interest to investigate whether changes in the concentration of adrenal corticosterone occur 2 or 3 hours after a single injection of ACTH, adrenaline or histamine. Results obtained in such an experiment are presented in Table 41. The hormone stores of the adrenal cortex were found to be increased by about 100% 2 and 3 hours after a single subcutaneous injection of adrenaline or ACTH. In contrast, 20 mg of histamine per kg body weight were without effect on the corticosterone concentration in the adrenals, although this quantity is reported to be sufficient to rapidly decrease the concentration of ascorbic acid in the gland

The results are summarised in Fig. XV.

TABLE 41

The effect of a single injection of ACTH, adrenaline or histamine on the corticosterone stores in rat adrenals.

Group a) Controls: 0.2 ml 0.9% saline s.c. 2 hours before rapid decapitation.
Group b) ACTH: 1 i.u. long acting ACTH-Z/rat s.c. 3 hours before rapid decapitation.
Group c) Adrenaline: 200 µg L-adrenaline base/kg b.wt. s.c. 2

Group	Date	No. of Expt.	Strain	Treatment	mean b.wt. rat (g)	No. of adrenal	adr. tissue (mg)
a)	4.12 1956	LXV	B	saline s.c. 2 hr before death	344	4	83.6
b)	4.12 1956	LXVI	B	ACTH s.c. 3 hr before death	391	5	104.2
	4.12 1956	LXVII	B		346	5	90.3
c)	4.12 1956	LXVIII	B	adrenaline s.c. 2 hr before death	343	5	95.0
	4.12 1956	LXIX	B		346	5	90.8
d)	4.12 1956	LXX	B	histamine s.c. 2 hr before death	364	5	93.4
	4.12 1956	LXXI	B		360	5	108.8
e)	1.3 1957	LXXII	B	histamine i.p. 10 min. before death	302	12	207
f)	1.3 1957	LXXIII	B	saline i.p. 10 min. before death	300	12	227

TABLE 41

hr before rapid decapitation.

Group d) Histamine: 20 mg histamine acid phosphate/kg b.wt. s.c. 2 hr before rapid decapitation.

Group e) Histamine: 20 mg histamine acid phosphate/kg b.wt. i.p. 10 min. before rapid decapitation.

Group f) Controls: 0.3 ml saline i.p. 10 min. before rapid decapitation.

No. of Expt.	µg adr./ kg b. wt.	Corticosterone store		% recovery	NaOH-fluor-escence
		µg/g adr.	µg/kg b.wt.		
LXV	122	14.4	1.75	79	x:?
LXVI	107	26.8	2.87	93	x:+ A:?
LXVII	104	28.8	3.0	71	x:-
LXVIII	111	36.9	4.08	74	x:+ A:?
LXIX	105	29.7	3.12	64	x:+
LXX	103	9.6	0.99	80	x:-
LXXI	121	14.7	1.78	92	x:?
LXXII	114	53.7	6.1	73	x:+
LXXIII	126	19.3	2.5	72	x:?

The failure of histamine to increase adrenal corticosterone 2 hours after it had been injected subcutaneously suggested a time course in the ACTH release which is different from that following an injection of adrenaline. Therefore an experiment was carried out in which adrenal corticosterone was measured 10 min. after an intraperitoneal injection of histamine. Twelve male rats (see Table 41, groups e and f) were accustomed to injections by giving them 1.0 ml/kg sterile saline intraperitoneally twice daily for 4 days. On the fifth day, 6 rats were given 20 mg/kg histamine acid phosphate (pH 7.4) intraperitoneally, the remaining rats the same volume of 0.9% NaCl. Exactly 10 minutes after the injection the rats were killed by rapid decapitation and the corticosterone concentration in the pooled adrenals of each group was estimated. It was found to be 53.7 µg/g adrenal in the glands obtained from the histamine-injected rats and 19.8 in the glands obtained from the saline controls.

Discussion

The foregoing studies on the corticosteroids extractable from rat adrenal tissue have shown that only those compounds are present which are also present in rat adrenal vein blood. None of the postulated C_{21}-precursors could be detected by the methods employed (see also p. 42).

The corticosteroids extractable from fresh adrenal tissue are genuinely "stored" in the gland tissue itself. The corticosterone, present in the amount of blood contained in the tissue could by no means account for the quantities found in the glands. Even if the blood content of an adrenal amounted to 20% of its weight, no more than 3 µg corticosterone per g tissue could be found under conditions of maximal secretion.

Quantitative estimations of the corticosterone concentration in rat adrenals confirm that the adrenal cortex stores only very small amounts of its actual secretion products, as compared with its secretion rate (Vogt, 1943).

The low figures explain the difficulty of detecting corticosteroids histochemically in adrenal tissue. An adrenal contains at the utmost, 0.5 µg corticosterone in 1 mm^3. Thus, in a 20 µ section not more than 0.001 µg are distributed over an area

of 3 mm^2 cortical tissue. Even if corticosterone were only present in the zona fasciculata, in droplets of the size of 1/10th of each cell, the concentration would be lower than 0.06 $\mu g/cm^2$. This is still ten times less than the threshold of the most sensitive reaction for detection of steroids.

The quantitative results obtained indicate a correlation between the quantities of hormones present in the adrenal cortex and its secretory activity at any time. A high adrenal corticosterone concentration appears to be associated with a high secretory activity, a low one with a low secretory activity.

Direct evidence for this correlation was obtained by comparing secretion rate and stores of cortical hormones in normal rats, and in rats pretreated with hexoestrol or amphenone. All rats were subjected to anaesthesia and operative stress.

Hexoestrol pretreatment resulted in a 55% decrease of hormone stores as well as of hormone secretion per unit weight of adrenal tissue, whereas pretreatment with amphenone led to an approximately 100% increase of stores and secretion per kg body weight. In each instance, the amount of corticosterone present per g adrenal tissue was

found equal to the amount secreted over a 3 minute period.

Indirect evidence for a positive correlation between the amounts of corticosterone present in an adrenal gland and its secretory activity derives from experiments, in which adrenals were obtained either shortly after rats were exposed to a brief, severe stress, or received a single injection of ACTH or adrenaline. The brief stress (exsanguination under ether anaesthesia) caused a nearly 4-fold elevation of the adrenal corticosterone concentration in all rats whether normal or pretreated for ten days with ACTH or amphenone. A single injection of adrenaline or ACTH was followed 2 or 3 hours later by a 2-fold increase of the corticosterone content of the adrenals. Ten minutes after injecting histamine adrenal corticosterone was nearly trebled. In all these examples, the mechanism is presumably the same: the adrenals are exposed to increased amounts of circulating ACTH for a short period. Ether plus rapid exsanguination was found to induce in the intact rat a significant rise in the blood concentration of ACTH (Sydnor and Sayers, 1954). An increase in the titre of blood ACTH after infusion of adrenaline into intact rats was observed by Farrell and Cann (1952) and by Gemzell (1952). The observation that 10 minutes

after histamine injection an increase in the adrenal corticosterone concentration occurred, but none two hours after the injection, is in good agreement with the findings of G. Sayers (1957). He measured the blood ACTH level after intraperitoneal injections of histamine and found it increased during a period of 5 to 14 minutes following the injection, but there was no elevation two hours later.

These findings suggest that stimulation of the adrenal cortex by ACTH leads to an elevation of the corticosterone concentration in the adrenal, provided steroid synthesis is not impaired. They further provide a method, consisting in the estimation of the corticosteroid content of adrenal tissue by which information can be obtained on the activity of the adrenal cortex immediately before extirpation of the gland.

Summary

1. Qualitative and quantitative studies on the corticosteroids extractable from rat adrenals were carried out.

2. Only compounds could be detected in the glands which are also present in rat adrenal vein blood.

3. The amount of corticosterone found in adrenals of "unstressed rats" of different strains ranged between 7.4 and 28.6 µg/g tissue.

4. These figures increased to 48.4 - 87 µg/g tissue when the rats were exposed to a brief period of severe stress before extirpation of the glands.

5. Pretreatment with hexoestrol for 5 days diminished the corticosterone stores in adrenals of "stressed rats" by about 50%.

6. Pretreatment with ACTH or amphenone "B" for 10 days caused an increase in the corticosterone stores per kg body weight, but not per g tissue.

7. Adrenal corticosterone concentration is doubled 2 hours after a single injection of adrenaline and 3 hours after one injection of ACTH; it is nearly trebled 10 minutes after an injection of histamine.

8. The relation between the corticosterone concentration of an adrenal gland and its secretory activity at any given time has been discussed.

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